2013

Pickering Stabilization of Oil-Water Interfaces by Heated B-lactoglobulin/Pectin Particles

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Graduate School ETD Form 9
(Revised 12/07)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

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By Laura Kathryn Zimmerer

Entitled
Pickering Stabilization of Oil-Water Interfaces by Heated B-lactoglobulin/Pectin Particles

For the degree of Master of Science

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Approved by: Chandrasekaran Rengaswami 10/04/2013
Head of the Graduate Program Date

PICKERING STABILIZATION OF OIL-WATER INTERFACES BY HEATED B-LACTOGLOBULIN/PECTIN PARTICLES

A Thesis

Submitted to the Faculty

of

Purdue University

by

Laura Kathryn Zimmerer

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

December 2013

Purdue University

West Lafayette, Indiana

ACKNOWLEDGEMENTS

I would first like to thank my professor, Dr. Owen Jones, for his guidance throughout my project. I've learned a tremendous amount and it was all thanks to his knowledge and expertise. Thank you to my committee members, Dr. Yuan Yao, Dr. Fernanda San Martin-Gonzalez, and Dr. Scott Peterson, I appreciate your feedback along this journey. The support of my family, especially my parents who never doubted me, has been a source of encouragement and inspiration. To my labmate and friend for life, Stacey Hirt, who was by my side from the beginning of grad school through the bitter end, we made it! Lastly, thank you to my gang back home who helped me to laugh when I needed it. Rachel, Liz, Anthony, Mark, Shane, Ty and Laura, you're the best friends anyone could ask for.

Reason will prevail!

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ABSTRACT

Zimmerer, Laura K. M.S., Purdue University, December 2013. Pickering Stabilization of Oil-Water Interfaces by Heated β-Lactoglobulin/Pectin Particles. Major Professor: Owen Jones.

The use of natural biopolymer particles as Pickering stabilizers for oil-in-water emulsions was investigated. β-lactoglobulin microgels and β-lactoglobulin/pectin complexes were created by heating appropriate biopolymer solutions at pH 5.8 and 4.75, respectively. Resultant particles exhibited spherical morphology with diameters of 100-300 nm and possessed significant negative surface charge. Particles were first homogenized with 1% corn oil at 0.05%, 0.1% or 0.25% (wt/ wt) particle concentrations. All emulsions appeared stable over seven days with only a thin, creamed ring forming after several hours. Corn oil emulsions were most stable with 0.25% heated complexes, with volume-weighted mean droplet diameter remaining around 480 nm throughout seven days of storage. When the 0.25% heated complexes were dialyzed to remove the free, unaggregated protein, the corn oil emulsions that resulted initially had similar volume-weighted droplet diameters as the undialyzed emulsions until day three of storage when the emulsions created with dialyzed complexes began to grow in size, reaching over 1 micron at day seven of storage.

In order to examine the effects of particle stabilized-emulsions on volatilization rates, 1% of a 90:10 blend of limonene:corn oil was homogenized with 0.25% heated complexes. Again, turbid emulsions resulted with no visible creamed ring and a stable volume-weighted mean droplet diameter beginning around 620nm and growing to 800 nm at the end of the seven day storage. The heated complex particles displayed interfacial activity as indicated by increased surface pressure at a limonene: corn oil droplet interface by inverted pendant drop analysis. The unheated β-lactoglobulin/pectin complexes were able to quickly increase the surface tension and reach equilibrium. In contrast, the heated complex particles displayed two different slopes of adsorption, the first quickly increasing surface pressure while the second slope was a more gradual increase in surface pressure attributed to the larger aggregates. Headspace gas chromatography revealed that after one day, no significant difference was seen in limonene headspace concentration as compared to control emulsions stabilized by the commercial surfactant Tween 20.

CHAPTER 1. INTRODUCTION

1.1 Introduction

The food industry has increasingly employed the use of encapsulation systems to protect value added ingredients, such as bioactive compounds, probiotics, prebiotics, vitamins or even flavors[1–3]. One of the methods commonly used to impart protection and stability to these ingredients is to emulsify them within an oil phase. Emulsions are systems of oil dispersed in water that are stabilized at the interface by emulsifiers which lower surface tension and prevent the separation of the two immiscible phases. Emulsifiers are typically classified as either surfactants, which are small amphiphilic molecules, or as solid particles that reside at the oil-water interface. These solid particle-stabilized emulsions are known as Pickering emulsions[4].

Pickering emulsion are theoretically much more stable than surfactant-stabilized emulsions owing to their large particle sizes which make desorption from the oil-water interface practically irreversible[5]. Additionally, the solid particles at the interface can pack quite rigidly and provide a significant steric barrier with which to prevent coalescence. The thick barrier at the oil interface also serves to slow the rate of diffusion out of the oil droplet. In the case of volatile flavor oils, this could be very advantageous in terms of increasing shelflife and preventing flavor loss. The area of research involving Pickering emulsions has investigated several different types of solid particles including

silica, gold, silver, carbon and many more synthetic materials. Binks et al. has specifically demonstrated that silica nanoparticles are able to decrease the limonene volatilization rate as compared to the surfactant, sodium dodecyl sulfate[6].

For emulsion uses within the food industry however, it may be beneficial to employ particles which are composed of solely food-grade, natural ingredients. Within this restriction, research on Pickering emulsions is limited to few materials that show promise for future development. Investigations into quinoa starch granules[7], zein protein[8], chitin nanocrystals[9] and lactoferrin nanoparticles[10] have all been applied as stabilizers of oil-in-water (o/w) emulsions.

Of specific interest to our research group is the use of heated β-lactoglobulin microgels and heated β-lactoglobulin/pectin complexes as potential stabilizers of o/w emulsions. Before the thermal treatment, the microgels at pH 5.8 are in a dimer state whereas the complexes at pH 4.75 are in an electrostatic complex. However, heating causes the proteins to denature and promotes crosslinking and hydrophobic aggregation within the particles, thus forming a more compact, solid particle suitable for use as a Pickering stabilizer[11,12]. Both heated microgel and complex particles have been investigated thoroughly and are shown to exhibit promising characteristics such as solid structure, diameters approximately at 100-300 nm, a spherical shape and sufficient electrical surface charge[13,14]. Additionally, both particles are created from naturally-occurring materials and do not require the use of toxic reagents or chemicals to form them.

1.2 Objectives

The overall objectives of the following experiments were to understand how heated microgels or heated complexes could act as Pickering stabilizers of oil-water emulsion interfaces and to understand how these solid-stabilized emulsions could inhibit the volatilization rates.

The specific goals of the research were to i) initially characterize the heated β-lactoglobulin microgels and heated β-lactoglobulin/pectin complexes and ii) to apply these particles to corn oil emulsion in order to find most successful emulsion parameters and lastly iii) to use the most successful particle parameters with a volatile emulsion system containing limonene in order to see effects on surface pressure and volatilization. We hypothesize that the heated particles will produce stable emulsions that are able to reduce the volatilization rates of limonene as compared to other surfactant stabilized emulsions.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Many popular products currently on the supermarket shelves today consist of mixed systems of oil in water (o/w) or water dispersed in oil (w/o), such as mayonnaise, ice cream, salad dressings, margarine and countless others. Due to the immiscibility of these components, which causes them to separate into distinct phases, there is a need for additional ingredients which can stabilize the dispersed phase within the continuous phase. These are known as emulsifiers and can exist in the form of small, amphiphilic molecules, long chain polymers or even small particles. The specific type of emulsion stabilized by these small, solid particles are known as Pickering emulsions[4].

The current literature review will first investigate the formulation of these solid nanoparticles from naturally-occurring biopolymers and the diversity found within this field. Next, the necessary requirements to use these particles as emulsion stabilizers will be presented along with successful reports of food-grade Pickering emulsions. Lastly, the encapsulation of volatile flavor oils, more specifically, via the use of solid particles or globular proteins to stabilize and protect the flavor oils, will be presented.

2.2 Biopolymer Particles

Colloidal particles are becoming increasingly more common within food products today due to their ability to specifically modify foods in terms of rheology, flavor, appearance and nutritional benefits[15,16]. Another benefit of these particulates is that they can be crafted from virtually any type or combination of biopolymer, ranging from polysaccharides[17] like gum Arabic, pectin, or xanthan gum, proteins like β-lactoglobulin, lactoferrin[10], or zein[18] and can even be made to include fats such as crystallized monoglycerides[19] or glyceryl stearyl citrate[20]. This versatility lends itself to the design of particles which can be tailored to fit any need. The main aspects that must be considered when designing these functional particles include composition and structure, desired physicochemical properties and the particles' sensitivity to environmental influences such as pH, temperature or physical stresses[21].

2.2.1 Composition and Structure

A variety of components have been used to create nanoparticles in the past, including, but not limited to chemically modified silica[22,23], hydrophilic clay particles[24], carbon nanoparticles[25], silver[26] and gold[27]. Using these inorganic materials offers the benefit of being cost effective, readily accessible and easily altered to suit a variety of uses. However, in the food industry, it is desirable to have ingredients composed of completely food-grade materials which have already been approved for human consumption[28]. Shapes such as fibrils, coils, crystalline structures or spheres can all be crafted from different biopolymers. The size of these particles can range from the nano-scale (less than 100 nanometers (nm)), the micro-scale (between 100 nm and 0.1mm) and

even the macro-scale (greater than 0.1 mm). Particle sizes are typically represented by a mean particle diameter because the population may be quite polydisperse, meaning that a wide range of sizes exist within the sample.

The internal structure of a particle can also assume various forms. Homogeneous particles have a solid, uniform composition throughout the whole structure, while heterogeneous particles can exist as core-shell templates that are hollow, making them great candidates to encapsulate sensitive compounds inside[3]. In contrast, heterogeneous dispersions consist of two or more distinct phases that can also be used to incorporate materials within the structure. The internal structure of particles can affect the permeability, density, loading capacity and digestibility.

2.2.2 Particle Fixatives

Upon creation of these biopolymer particles, it may be necessary to further treat them to impart stability throughout further processing, storage and consumption. Several methods exist with which to stabilize biopolymers and make them resistant to unfolding or physical stress, including enzymatic crosslinking, chemical crosslinking, desolvation or thermal treatment. . Chemical crosslinking is oftentimes performed with glutaraldehyde which is not yet approved for consumption in foods because of toxicity concerns[29].

Thermal treatments are specifically advantageous because they require no expensive chemicals or toxic reagents and can be carefully controlled. As biopolymer particles are heated, denaturation can start to take place and to a certain extent, may be reversible. Once covalent bonds form between proteins themselves or between proteins

and polysaccharides however, the denaturation of the particle is irreversible. These forces are ideal for holding together the particle and creating a more solid, compact unit that can be placed at interfaces to lower interfacial tension, as will be discussed.

2.2.3 Soluble Complexes

Another unique aspect of these biopolymer particles that is becoming of increasing use is the ability to create molecular interactions between two or more different polymers. This interaction could involve electrical interactions, hydrophobic forces, covalent bonding or hydrogen bonding. Of specific interest to this project is the electrical interaction that can occur between two polymers with the manipulation to pH. At a pH value above a proteins isoelectric point (pI), the protein will have a net negative charge. As the pH value is decreased to slightly below the pI, the protein begins to take on a net positive charge. When in the presence of a complimentary polymer, such as an anionic polysaccharide, this can create an electrical interaction between the oppositely charged polymers thus forming a soluble complex[17]. For example, the protein β-lactoglobulin has been shown to create these complexes with anionic polysaccharides such as gum Arabic[30,31], pectin[32], κ-carrageenan[33] and sodium alginate[34]. Cationic polysaccharides can also be used to form complexes following the same basic principles except that the pH of the protein must be above the pI in order to create an electrical interaction between the positively charged polysaccharide and the negatively charged protein. Successful complexes have been formed this way between ovalbumin and chitosan[35], as well as between β-lactoglobulin and chitosan[36].

As stronger interactions between biopolymers occur, such as with low salt concentrations, pH further from the pI and higher charge density polymers, a coacervate rather than a soluble complex is formed. This coacervate is less hydrated than the soluble complexes mentioned above and is more compact, thus it is important to make the distinction between the two[37]. However, with applied heat treatment to the soluble complexes, the structure becomes more compact and rigid in order to form more of a particle rather than a loose association. As these coacervate interactions become even stronger, a precipitation and phase separation will result yielding a coacervate-rich lower phase and a coacervate-poor serum phase.

2.2.4 Microgels

If a system is composed of solely protein and no polysaccharide, it is still possible to create biopolymer particles through specific protein concentrations, pH, ionic strength and heating conditions. These soft, spherical colloids are formed by weak crosslinking of protein chains and are known as microgels[38]. Heat treatments are the most typical way to form microgels because during the denaturation step, proteins unfold to expose side groups which can readily crosslink via covalent interactions of disulfide groups or by hydrophobic interactions. Above a certain critical gel concentration, heating the proteins will form a gel rather than particulates. This gel concentration is typically lower with lower electrostatic repulsion between proteins[39].

In the case of the protein β-lactoglobulin, a specific pH range from below 7.0 to slightly above the pI of 5.2 is needed along with low ionic strengths to keep the protein solubilized. If the protein were heated near its pI when the net charge is close to neutral,

aggregates would precipitate out of solution because there is not enough electrostatic repulsion between the proteins[16,40]. Donato and others found that solutions of β-lactoglobulin heated at pH 7.0 remained transparent while those solutions heated at pH 5.7 turned turbid and white suggesting aggregation[38]. Micrographs of β-lactoglobulin solutions heated at 85^{O}C and pH 5.9 indicated spherical aggregates with an average diameter of 200 nm. As heating time increased, the particle shape became more ellipsoidal while polydispersity decreased.

Similarly, whey protein isolate at pH 6.0 has been shown to form soluble aggregates upon heating for 15 minutes at 85^{O}C.[41] One unique phenomenon that occurs upon formation of β-lactoglobulin microgels is a spontaneous rise in pH which inhibits secondary aggregation, leading to a stable suspension[42]. This rise in pH was found to only occur if the initial pH of solution was between 5.75 and 6.1, reinforcing the idea that the pH range is a critical parameter in stable microgel formation. If pH values are not in the desirable range for microgel formation, the addition of calcium chloride salts has been shown to aid in formation of β-lactoglobulin microgels when above a certain critical gel concentration whereas when no $CaCl_2$ is added, heating at pH 7.0 results in small strands of denatured protein.[43]

2.2.5 Biopolymers for Particle Formation

It is important to understand the individual components that are intended to create these biopolymer particles and ensure that they are both compatible for one another and suitable for each application. Bovine β-lactoglobulin is arguably one of the most studied food proteins used in biopolymer fabrication. β-lactoglobulin is the predominant protein

of the two major protein fractions in whey; the other fraction being α-lactalbumin. It is globular in nature with a molecular weight of approximately 18.3 kilodaltons, radius of 2nm and melting temperature of 75^{O}C[44]. In solution, the protein exists as a mixture of both monomers and dimmers, but as temperature is increased, the equilibrium shifts towards the monomer form[45]. Nine antiparallel β-sheet protein strands coil around to create a barrel shape that is closed on one end. Upon heating, this globular, tertiary structure unfolds to expose a hydrophobic cavity which is a potential site for ligand binding[46]. Two disulfide bonds and one free thiol exist within β-lactoglobulin which can also become available for reaction or cross-linking upon denaturation and unfolding[44].

Upon heating, β-lactoglobulin will start to denature. As with most proteins, denaturation begins with loss of the quaternary structure first, followed by the unraveling of the secondary and tertiary structure[47,48]. This can help to expose interior hydrophobic groups that have potential to create disulfide bonds or crosslinks between other polymers in solution[49]. For β-lactoglobulin specifically, the interior thiol group can become exposed during this molten-globule state[50]. Additionally, once the hydrophobic groups are exposed during heating, there is an increase in the hydrophobic association driving aggregation[51].

The isoelectric point (pI) of β-lactoglobulin has been found in the range of 4.8-5.2 depending on solution conditions and protein purity[44,46]. While at a pH above the pI, the protein has a net positive charge and near the pI, the protein takes on an overall neutral charge which can be seen by the precipitation that often occurs at the pI. When the charge becomes neutral, there is little to no electrostatic repulsion within the protein so it aggregates and falls out of solution. Below the pI, the protein takes on a net positive

charge. This aspect of altering charge can be specifically manipulated to create interactions within the protein itself or with other polymers, such as anionic polysaccharides[52].

One such commonly used polysaccharide is pectin. Pectin is a plant cell wall polysaccharide composed of α-(1,4)-covalently-linked galacturonic acid units interrupted by occasional rhamnose sugar residues[53,54]. Varying degrees of carboxylation on the galacturonic acid units can change the degree of esterification (DE) from a low DE value of 0 to a high DE value of 100. A DE value less than 50 is known as low-methoxy (LM) pectin while those pectins with DE values greater than 50 are known as high-methoxy (HM) pectins[55]. The DE value of the pectin can directly affect functional properties such as gelling and emulsifying capabilities so it is an important factor to keep in mind when selecting the appropriate pectin for a specific use. HM pectin gels at low pH and high sugar contents, while LM pectin gels at higher pH values in the presence of multivalent cations, like calcium or magnesium[56]. Notably, pectin has been shown to stabilize emulsions better and at a lower dose than another common, food grade polysaccharide, gum Arabic[57]. However, this is likely due to the small fraction of protein associated with the pectin. Additionally, it was found that sugar beet pectin had more favorable emulsion stabilizing properties than citrus pectin, likely due to a larger protein and acetyl content or its more relaxed conformation.

Pectins are largely sourced from apple pomace and citrus skins as a by-product of processing. However, recently other sources of pectin from sugar beets and sunflowers have been utilized[58]. The pectin is typically extracted from the fruit or plant matter via acid extraction and then precipitated with aluminum salts or alcohols.

2.2.6 Particle Electrical Characteristics

The electrical characteristics of a biopolymer particle can influence several things, most importantly how it reacts in solution in relation to other particles. For example, if particles are all highly charged, whether negative or positive, they should repel one another and remain in solution. This is important to prevent aggregation and maintain distinct particles rather than flocculates. The surface charge of nanoparticles is commonly measured in terms of electrostatic surface potential, or ζ-potential[59]. The surface potential is related to the counterion layers surrounding the particle itself and is a direct measure of the response to an applied electrical field.

Changes to pH or ionic strength can effectively change the electrical characteristics of a particle by ionic screening effects. With increases to the ionic strength of the solution medium, ions screen the charges and diminish the repulsive forces. Guzey et. al. found that corn oil emulsions stabilized by β-lactoglobulin were destabilized by salt additions prior to pectin adsorption whereas salt additions after pectin deposition onto the protein helped to stabilized the emulsion system[60]. This behavior was attributed to the ability of the NaCl to promote droplet flocculation.

2.3 Solid-Stabilized Emulsions

The term emulsion is broadly defined as droplets of an immiscible liquid dispersed in another liquid phase. Emulsions can exist in a wide range of sizes, including microemulsions of droplet radius less than 10nm, nanoemulsions that have droplet radii less than 100nm and macroemulsions that are the largest at 100 to 10,000 nm in droplet diameter[61,62]. Therefore, in order to prevent the immiscible phases from separating,

stabilizing techniques must be employed. One way to impart stability to an emulsion is by creating a Pickering emulsion, sometimes known as a colloidosome although this terminology is more often used to describe supra-colloidal hollow spheres where the dispersed phase of a solid-stabilized emulsion had been removed.

In a Pickering emulsion, solid particles adsorb to the interface between the two phases forming a monolayer[4,63]. This is distinctly different from a typical surfactant stabilized emulsion in which a flexible polymer (protein or polysaccharide) or an amphiphilic surfactant attaches to the oil-water interface. Once inserted into the interface, the particles lower interfacial tension and raise the droplet surface pressure. The size and coverage of the interfacial particles must be sufficient enough to prevent flocculation, coalescence or film drainage. The importance of particle concentration and oil droplet coverage will be covered in more detail in future sections.

As mentioned previously, it is advantageous to create products with food-grade ingredients to avoid any regulatory or health issues. Relatively little work has been done in regards to solid particle-stabilized emulsions composed of natural and edible ingredients. Research thus far includes work on emulsions stabilized by starch granules isolated from quinoa[7], zein nanoparticles[64], lactoferrin nanoparticles[10], solid lipid nanoparticles composed of glycerin tristearate[20], and esterified cotton cellulose nanowhiskers[65].

2.3.1 Requirements of the Particles

In order to be suitable for use as a Pickering stabilizer, particles must exhibit certain qualities including dual wettability in both phases and a solid and uniform

composition, implying that they are free of surfactants or other potential contaminants. Particles intended for use as Pickering stabilizers should also ideally be significantly larger than classical surfactants. Arguably, the most important aspect of the particles is their wettability, which is an indication of the contact angle that the particle makes between the oil and aqueous phases[66,67]. Hydrophilic particles tend to have contact angles less than 90^{O} in oil-in water systems, meaning that the majority of the particle exists in the aqueous phase rather than being inserted into the nonpolar oil phase. This type of particle would tend to stabilize or promote water-in-oil emulsions. In contrast, hydrophobic particles would have a tendency to reside further into the oil phase and therefore, would exhibit contact angles greater than 90^{O} so would be more likely to form oil-in-water emulsions[5,68]. The most common example of this would be the "water-loving" silica particles which tend to stabilize oil-in-water emulsions[69].

With the solid composition also comes the requirement that the particles be of a larger diameter than typical surfactant molecules. As shown in Figure 2.1, Binks et al. clearly demonstrate that the radius of a particle at an interface will directly affect the energy of attachment[5]. We see that larger particles, as found with Pickering emulsions, have much higher energy requirements in order to adsorb to the interface but once there, the particles are effectively irreversibly adsorbed and contribute to a very stable emulsion. As the contact angle approaches 0^{O} or 180^{O} though, the particles become less stable at the interface[66]. When particle diameters reach very small sizes (less than 0.5nm), similar to sizes of surfactants, this energy of adsorption becomes very low, and therefore, the particles can detach from the interface quite quickly and easily.

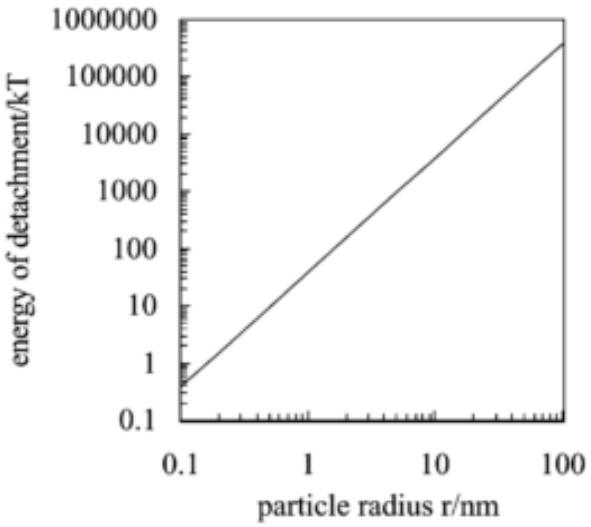

Figure 2.1 Plot of the effect of particle radius of a single spherical particle with contact angle of 90^{O} on the energy of detachment from an oil-water interface of 50mN/m interfacial tension[5].

Li et. al. found that when using native potato starch granules ($d_{4,3}$ of 52.1 μm) however, the larger particles sizes did not form emulsions whereas the smaller native rice starch granules ($d_{4,3}$ of 5.2 μm) were able to form stable emulsions throughout several months[70]. This could be explained by the much high amount of potato starch needed for sufficient surface coverage as opposed to smaller particles of rice starch which can also pack more efficiently at the interface.

In order to be classified as a Pickering stabilizer, particles must also be solid and of uniform composition[66]. For example, globular proteins, like native β-lactoglobulin, have a tendency to unfold at the oil-water interface in order to insert hydrophobic regions of the protein chain into the nonpolar phase while the charged, hydrophilic portions of the protein would tend to lie in the aqueous phase[71]. The loss in tertiary structure during the unfolding process at the interface would not fall under the classification of a Pickering stabilized emulsion because the protein is now unfolded and not acting as a particle.

Additionally, the particle should be homogenous in composition throughout its entirety, including being free of other small-molecule surfactants which would be the predominant interfacial stabilizers rather than the larger Pickering particles[66]. When surfactants and particles are present in an emulsion system, it has been demonstrated that both Span 80 and Tween 20 can displace the globular protein, β-lactoglobulin, from the interface[72] while lecithin and sodium dodecyl sulfate have been shown to replace silica particles at an oil interface[73,74].

2.3.2 Factors Affecting Pickering Emulsion Stability

Once the biopolymer particles have been successfully created, inserting them at the oil-water interface is the next challenge that must be overcome. Considerations that should be made include particle concentration, which impacts interfacial packing structure, ionic strength of solution and oil type.

The ratio between the particles and the oil content directly affects the droplet size and stability of the emulsion. As particle concentration is increased, coverage of the oil droplets becomes more complete and allows for stabilization of smaller droplets. Kargar et. al. found that with both micro-crystalline cellulose and modified starch particles, an increase in particle concentration from 0.5% to 2.5% resulted in a decreased droplet size of sunflower oil covered droplets[75]. Additionally, this aided in the creaming stability of both emulsions. The increasing particle concentration helped to increase the rate of particle adsorption at the oil interface, as well as increasing the viscosity of the whole system. When insufficient particle concentrations are used, oil droplets are more prone to

coalescence because of a lack of steric barrier and electrostatic repulsion from the interfacial particles themselves.

Emulsion droplet diameters are typically represented as either a surface-weighted mean diameter ($d_{3,2}$), which tends to emphasize the population of smaller droplets, or a volume-weighted mean diameter ($d_{4,3}$) which will skew more towards the larger droplet sizes[76]. For some purposes, it is helpful to include both values to obtain a more complete and accurate depiction of the population distribution. The following formulas are used to calculate the various mean droplet size diameters where d_i is the diameter of the i^{th} droplet[77].

$$d_{3,2} = \Sigma d_i^3 / \Sigma d_i^2 \quad (1)$$

$$d_{4,3} = \Sigma d_i^4 / \Sigma d_i^3 \quad (2)$$

Stability of emulsions can also depend on the type of oil in the dispersed phase. A wide range of solubility, molecular weight and hydrophobicity can be found across different flavor oils. For example, the composition of lemon oil can change depending on the "fold" or distillation procedures that it undergoes. A single fold, or cold press, lemon oil is higher in compounds with low molecular weight. However, as the fold increases to the likes of 4x or 10x and thus, undergoes more distillation, the lemon oil becomes increasingly higher in larger molecular weight compounds[78]. This compositional variation can directly affect the droplet size of an emulsion over storage time by either decreasing droplet size through solubilization into micelles or growth via Ostwald ripening.

All of these factors are important in order to create the most stable emulsion but it is also important to understand how Pickering emulsions can be broken or destabilized. Destabilization can occur via sedimentation, flocculation, Ostwald ripening or coalescence. Sedimentation occurs when emulsion droplets precipitate to the bottom or cream to the top of the emulsion, while with flocculation we see that droplet integrity is maintained but the droplets aggregate together in large flocs[76]. Coalescence is a phenomenon that occurs when two droplets of comparable size collide and form one larger droplet. Ostwald ripening is a similar phenomenon but specifically involves the loss of smaller oil droplets at the expense of larger droplets. This is due to the increased surface pressure found with smaller, high curvature droplets of oil. The higher pressure forces the oil to become more soluble in the aqueous phase which then allows the solubilized oil molecules to travel through solution into a larger oil droplet with less surface pressure[79]. At low concentrations of silica nanoparticles, the initial destabilization was observed to be a combination of flocculation and permeation of the oil phase from smaller droplets to nearby larger ones, the aforementioned Ostwald ripening.[80] At higher particle concentrations, the silica forms a loose network which works to stabilize the droplets and keep them well separated. If the oil droplets are kept far apart, there is less likelihood that pores would form between the two droplets or that the oil would migrate into larger droplets.

Many studies have been conducted which prove that the oil phase composition can directly affect the rates and extent of Ostwald ripening in an emulsion, whether it is by varying polarities of the oil[81], triglyceride content[82] or solubility of the oil in the aqueous phase[83]. For example, McClements found that by incorporating at least 10% corn

oil into the citrus oil prior to homogenization, droplet growth could be halted[84]. This is attributed to the fact that Ostwald ripening is opposed by the compositional ripening effect. Compositional ripening occurs when oil droplets contain a mixture of two different oils, for example, a water-soluble oil and water-insoluble oil, and the droplets have different oil compositions. If one droplet contains a relatively high concentration of the water-soluble oil as compared to a second droplet which contains a low concentration of the water-soluble oil (and therefore a high concentration of the water-insoluble oil), the water-soluble oil will distribute itself so as to even out the concentration gradient. Through this movement of molecules due to concurrent Ostwald ripening and compositional ripening, droplet growth can be inhibited. For compounds, such as the relatively water-soluble limonene which are prone to Ostwald ripening, a blend of oils could potentially be used to stabilize the emulsion system. It has also been demonstrated that addition of ester gum, a highly hydrophobic ester gum used as a weighting agent, can prevent Ostwald ripening in flavor oil-in-water emulsions[85].

Changing the pH of the Pickering emulsion, both before and after emulsification, can be used as a trigger to destabilize the system. Dyab showed that silica stabilized oil-in-water emulsions were especially sensitive at pH 2.94, the isoelectric point of silica, and also at pH 7.1 resulting in creaming and oil loss [86]. The emulsions that were adjusted prior to emulsification showed complete destabilization in the range of pH 7-8. The likely cause of instability is due to the negative effects of pH on the wettability of silica and therefore its contact angle with the oil phase. Diluting the emulsion system with added salt or surfactant has also been shown to affect creaming rates [87]. The silica Pickering emulsions responded positively towards additions of NaCl solutions with decreased

creaming rates. When solutions of the anionic surfactant, sodium dodecyl sulfate, were used to dilute the Pickering emulsion in small amounts, no significant changes in stability was noted. At additions above the critical micelle concentration however, rapid creaming and flocculation were observed. The excess surfactant interacted with the emulsion drops to cause depletion flocculation.

In a concurrent study, Whitby et. al. demonstrated that this system at rest did not coalesce. Upon shearing, the silica nanoparticle network is disturbed allowing for increases in droplet collisions and therefore coalescence[23]. They also demonstrated that the increasing salt concentration was able to diminish the electrostatic repulsive interactions between particles which results in a thicker interfacial layer of aggregate clusters. This, in turn, imparts greater stability to shear induced coalescence.

2.4 Encapsulation of Volatiles

Oftentimes, flavor compounds are added to foods in very low levels but are not perceived until released into the headspace where they can be smelled or when they are released from the food matrix upon chewing to be tasted. It may be of interest to delay this release in order to tailor the sensory characteristics of a food or to prolong the shelf life of the product. Typically, flavors are small molecular weight compounds which are added in very small amounts due to their low chemical threshold. Limonene, for example, has an odor threshold at 15 ppb in air[88], while the orthonasal threshold in water is found to be 10 ng/L[89]. In order to understand the capability of these Pickering emulsions to protect components or slow release, we must first understand the thermodynamics and kinetics of the emulsion system.

2.4.1 Thermodynamics of Flavor in Food Emulsions

Within an emulsion, a flavor or aroma compound will distribute itself so that chemical potentials are equal across all phases. These would include the dispersed oil phase, aqueous phase and the headspace above the emulsion. Additionally, the ratio of the chemical compound will differ throughout each phase depending on the affinity of the molecule for each phase[90]. A value known as a partition coefficient (K) can indicate the thermodynamics of aroma molecule binding (Equation 3).

$$K_{gp} = c_g / c_p \quad (3)$$

Where K_{gp} is the partition coefficient between a food product and the gaseous headspace, c_g is the aroma concentration in the headspace and c_p is the aroma concentration in the product.

Flavors and/or aromas with a higher K value will be more readily released from the food matrix and therefore, will be perceived more intensely than those aromas with a lower K value which display a high affinity for the food. The perception of a flavor from an emulsion is largely a factor of its concentration in headspace and the rate at which it is released, which in turn can be directly altered via the emulsion parameters[2,91,92]. Factors such as changes in oil droplet diameter, oil content and type, interfacial coverage with emulsifiers and dissolved solids within the aqueous phase can all be manipulated to alter the partition coefficient of an aroma[81,92–94]. Although the thermodynamics of aroma release will indicate in which direction the molecules should theoretically move, this tells us nothing about the relative timescale with which it will do so. For information on how quickly this partitioning and release will occur, we must understand the kinetics of an emulsion system.

2.4.2 Kinetics of Flavor Release from Emulsions

Distribution of an aroma molecule can change throughout the shelf life of a product, ranging from initial mixing, when a food in chewed in the mouth, as it is chewed and as saliva mixes with the product. In each of these steps, the matrix is changing which in turn, alters partitioning behavior and the concentration of molecules available for sensory perception, whether than be taste or smell[95].

In order for an aroma compound to even enter into the headspace, several barriers must be crossed beginning with mass transport out of the oil droplet, then transport across the interfacial barrier, diffusion through the aqueous phase and lastly, movement across the water-air interface, commonly termed the boundary layer[90]. In the case of Pickering-stabilized emulsions, the most significant barrier to volatilization would be at the oil-water interface and would therefore, be the rate limiting step[93]. Depending on the interfacial thickness and packing structure, particles at the interface should provide a significant barrier to impede the mass transfer from out of the oil droplet. Emulsions can also inhibit volatilization by thickening the aqueous phase with water-binding hydrocolloids, thereby slowing diffusion,[96] or by physically binding the volatiles within proteins, such as in the hydrophobic pocket of β-lactoglobulin[97].

Relatively little other work with volatile release has been performed because most studies employ the use of non-volatile oils such as hexadecane or corn oil to study the effects of Pickering stabilized emulsions. In one interesting study, Wang et. al. used Pickering emulsions stabilized with calcium carbonate crystals to act as a template for forming rigid capsules that were used to encapsulate a blend of sunflower oil with 20% limonene[98]. Upon further crystallization, these Pickering templates became rigid and

were able to be dried. Using proton transfer reaction- mass spectrometry (PTR-MS), it was demonstrated that that dried capsules dissolved in neutral water showed a delayed release profile of limonene to around 40 minutes whereas the pure limonene solution exhibited a release profile that diminished after only 5 minutes. The effects on evaporation rate of o/w limonene emulsions were studied by Binks et. al[6]. It was found that silica nanoparticles were able to more successfully inhibit the release of limonene as compared to a surfactant film of SDS. Additionally, they found that further decreases in volatilization rates could be achieved when the nanoparticle film was compressed by preshrinking the oil droplets. These trends were less apparent for benzyl acetate, which has a much higher solubility in and affinity for the aqueous phase.

Thermodynamically, foods that exhibit a greater affinity for the aroma molecule will release fewer aromas into the headspace for detection. Kinetically, however, release is mostly affected by the food structure itself and which aspects of the emulsion structure are able to inhibit mass transfer.

2.5 Conclusion

Small, solid particles that are crafted from natural, food-grade biopolymers, such as proteins and polysaccharides, show great promise for use as Pickering stabilizers. Because they can easily be tailored in respect to structure, electrical charge, size and density, these particles can be applied across a wide range of uses, from pharmaceutical to food products. Pickering emulsions specifically offer the advantages of being stable throughout an extended shelf life, a benefit that can be especially advantageous when formulating food products or beverages. The larger, solid particles that stabilize the oil-

water interface are resistant to desorption, as opposed to small surfactants which can readily be displaced from the interface. Pickering emulsions may also be suitable to provide barriers against volatilization or degradation of aroma compounds.

CHAPTER 3. PICKERING STABILIZATION OF OIL-WATER INTERFACES BY HEATED B-LACTOGLOBULIN/PECTIN PARTICLES

3.1 Abstract

The goal of this research was to investigate the use of natural biopolymer particles as stabilizers for oil-in-water (o/w) emulsions. β-lactoglobulin microgels and β-lactoglobulin/pectin complexes were created by heating appropriate biopolymer solutions at pH 5.80 and 4.75, respectively. Resultant particles exhibited spherical morphology and ranged from roughly 100-300 nm in diameter with sufficient electrostatic repulsion. Particles were first homogenized with 1% corn oil at 0.05%, 0.1% or 0.25% (wt/ wt) particle concentrations. All emulsions appeared stable over seven days with only a thin, creamed ring forming after one day. Volume-weighted mean droplet diameters were most stable and consistent for the 0.25% heated complex-stabilized corn oil emulsions, remaining around 480 nm throughout seven days. When the 0.25% heated complexes were dialyzed to remove the free, unaggregated protein however, the corn oil emulsions that resulted had substantially larger mean droplet diameters, indicating less stability than those emulsions stabilized by the undialyzed complexes.

In order to examine the effects of particle stabilized emulsions on volatilization rates, 1% of a 90:10 blend of limonene:corn oil was homogenized with 0.25% heated complexes. Again, stable emulsions resulted with no visible separation and a stable volume-weighted mean droplet diameter of approximately 300nm. The heated complex

particles were able to display interfacial activity by increasing surface pressure of a limonene: corn oil droplet by inverted pendant drop analysis. However, different profiles of adsorption were seen between heated and unheated particles. Headspace gas chromatography revealed that after one day however, no significant different was seen in limonene headspace concentration as compared to control emulsions stabilized with either 0.05% Tween 20 or 0.5% Tween 20.

3.2 Introduction

Stabilization of oil-liquid interfaces can be achieved by solid particles, thus forming what is termed "Pickering emulsions". These emulsions are specifically advantageous as compared to surfactant-stabilized emulsions in that the interfacial particles form a large, thick barrier which can contribute stability against coalescence and also has the potential to slow the rate of oil diffusion out of the oil droplet[5]. Particles with suitable wettability are able to reside at the oil-water interface in order to lower interfacial tension of the droplet. These solid particles are practically resistant to desorption once at the interface because of the large energy input required to remove them thus making them advantageous over conventional surfactant-stabilized emulsions[99].

Over the last few decades, a resurgence in interest of Pickering emulsions has occurred. Many studies have investigated the effects of particle size and shape, hydrophobicity, and oil type on the stability and mechanisms of these solid-stabilized emulsions[81,100–103]. Currently the challenge is to employ food-grade ingredients as stabilizers, rather than synthetic materials. Therefore, it is our desire to investigate the use of natural particles composed of biopolymers as Pickering-type stabilizers. Here we

introduce the prospect of heated β-lactoglobulin microgels at pH 5.8 and heated β-lactoglobulin/pectin complexes at pH 4.75 as viable alternatives to inorganic nanoparticles. The protein, β-lactoglobulin, and the polysaccharide, pectin, are very well researched and are sourced from edible products. By manipulating pH of the solutions prior to heat treatment, solid particles can be fabricated[38,104]. We demonstrate the fabrication of these heated microgel and complex particles as well as their efficacy as stabilizers of corn oil-in-water (o/w) emulsions. Additionally, we investigate the effects of varying particle concentration, the presence of free, unaggregated protein and oil composition on the stability of these particle-stabilized emulsions throughout a seven day period. The ability of these heated complex particles to increase surface pressure of oil droplets and to slow the rate of volatile limonene diffusion out of the o/w emulsion was also studied.

3.3 Materials and Methods

3.3.1 Materials

Citrus pectin was donated by CP Kelco (Atlanta, GA, sample # 3675-DE-52) and was reported by the manufacturer to have degree of esterification (DE) of 52%. No further purification procedures were used. Solid sodium hydroxide, sodium acetate, concentrated 12.1 normal (N) hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), R-(+)-limonene and the R-(+)-limonene analytical standard were all purchased from Sigma Aldrich (St. Louis, MO). Corn oil was purchased from a local retailer (Great Value, Wal-Mart, Bentonville, AR). All water used in solutions was purified through a

Barnstead E-pure 3 module water purification system (Thermo Scientific, Waltham, MA) with resistivity of 18 mW/cm.

β-lactoglobulin powder was provided by Davisco Foods International (Le Seuer, MN, lot # JE 001-0-415). Reported composition from the manufacturer was 97.9% protein with 91.5% of that being β-lactoglobulin, 0.2% fat, 1.8% ash, and 4.4% moisture. Dialysis procedures were used to purify the protein in order to remove excess denatured proteins, ions and impurities using the method of Jung et. al. [105]. In brief, a 10% (wt/wt) β-lactoglobulin solution was made with ultrapure water and adjusted to pH 4.60 using 3, 1, 0.1 and/or 0.01 normal (N) HCl solutions. This solution was then centrifuged at 15,000 rpm for 20 minutes using a Beckman Coulter JA-14 centrifuge (Brea, CA) with a regulated temperature of 20^{O}C. The supernatant was retained while the insoluble, denatured protein pellet was discarded. Aqueous solutions of 1, 0.1 or 0.01N sodium hydroxide were then used to adjust the resulting supernatant to pH 7.00. Spectra/Por membrane tubing with molecular weight cut off from 6,000-8,000 Dalton was boiled for one minute in 1 millimolar (mM) ethylenediaminetetraacetic (EDTA) acid solution and thoroughly rinsed with ultrapure water. The dialysis tubing was filled with supernatant at a 40:1 (vol/vol) ratio of protein solution:dialysis water. Dialysis water was replaced with fresh, ultrapure water after 1, 2, 4, 12, 24, 24 and 24 hours. Once the dialysis procedure was complete, the retentate was collected in 50 mL plastic centrifuge tubes and frozen for 24 hours. The frozen protein solution was then lyophilized for eight days until completely devoid of moisture. Purified, freeze-dried β-lactoglobulin was stored in the freezer at -4^{O}C until further use.

3.3.2 Preparation of Stock Particle Solutions

In order to create the complexes, solutions of 0.5% β-lactoglobulin (wt/wt) and 0.25% pectin (wt/wt) were prepared from the solid form and dispersed within 10mM sodium acetate buffer at pH 7.00 by stirring for at least four hours at 25^{O}C temperature or until completely dissolved. Equal weights of protein and polysaccharide solution were combined in a separate vessel to achieve a final concentration of 0.25% β-lactoglobulin (wt/wt) and 0.125% pectin (wt/wt). Mixed biopolymer solutions were then adjusted to pH 4.75 while stirring at 220 rpm using a Fisher Scientific pH meter (Columbus, OH) and dropwise additions of 3, 1 or 0.1N HCl solutions. Heat treatments were performed by submerging sealed flasks in a Grant GD100 water bath (Keison Products, Chelmsford, Essex, England) at 85^{O}C for 15 minutes. An ice bath was used to immediately cool the resulting stock solution until cool to the touch, which was stored under refrigeration until use.

In order to create the stock solutions of 0.25% β-lactoglobulin microgels, aqueous dispersions of 0.25% β-lactoglobulin (wt/wt) in 10mM sodium acetate buffer were prepared by weighing appropriate amounts of powdered protein and pH 7.00 buffer into a clean beaker. Once the solution had been allowed to solubilize for at least four hours on a stir plate, it was adjusted to pH 5.80 using drop wise additions of 1N and 0.1N HCl solutions. Heat treatments were performed by submerging sealed flasks in a Grant GD100 water bath (Keison Products, Chelmsford, Essex, England) at 85^{O}C for 15 minutes. An ice bath was used to immediately cool the resulting stock solution which was stored under refrigeration for further use. The effects of varying particle concentrations were studied by diluting stock solutions of complexes and microgels with pH 4.75 or pH

5.80 10 mM sodium acetate buffer, respectively, until particle concentrations reached 0.1% or 0.05% (wt/wt).

Portions of the stock complex solutions were subjected to a dialysis treatment in order to remove the free, unaggregated protein and pectin. This was performed by dialyzing the heat-treated, stock complex solutions against pH 4.75 10 mM sodium acetate buffer using a 40:1 (vol/vol) ratio of solution to buffer. Spectra/Por tubing (Spectrum Industries, Rancho Dominguez, CA) with molecular weight cutoff (MWCO) of 50 kDa was used in order to retain the larger, aggregated particles. Dialysis buffer was changed every 24 hours for one week and the entire procedure was performed at refrigeration temperatures while stirring at 120 rpm. Afterwards, the retentate inside the dialysis tubes was collected and used for further experimentation in emulsion trials. The protein concentration of the rententate was determined by direct absorption of ultraviolet-visible (UV-VIS) light at a wavelength of 280 nm using a Perkin Elmer Lambda 25 spectrophotometer (Waltham, MA). Stock solutions of heat-treated complexes that had not been dialyzed were used to create a standard curve. Absorption of visible light by the heat-treated β-lactoglobulin solutions at 280 nm is due to the presence of tryptophan and tyrosine residues and therefore, scales linearly with the quantity of protein [106]. The dialyzed stock solutions were diluted by 100x and the absorbance reading was fitted into the standard curve equation to obtain protein concentration.

3.3.3 Emulsion Preparation

Initial emulsion trials used 1% corn oil as the dispersed phase in order to see the effects of varying particle concentration beginning with the microgel solutions and

subsequently involving the complex solutions. In order to investigate the capability of these heat-treated particles to encapsulate flavor oils, limonene was used in combination with 10% corn oil for added stability to Ostwald ripening [84]. It was decided that for these trials, only the 0.25% stock solution of complexes would be used in this application because of the droplet size stability over time. Once again, the oil phase (90:10 limonene:corn oil) was added to the particle solution at 1% wt/wt. The complex emulsion of limonene with corn oil was compared to an emulsion of 1% 90:10 limonene:corn oil in 0.05% Tween 20 and also to an emulsion containing 1% 90:10 limonene:corn oil dispersed in 0.5% Tween 20.

Stock solutions of either complexes or microgels with the oil phase and/or buffer were then coarsely homogenized to incorporate the oil phase using a Polytron PT 2100 (Kinematica Incorporated, Bohemica, NY) at 22,000 rpm for 20 seconds. The resulting coarse emulsion was then finely homogenized using a high pressure pneumatic Microfluidizer, model 110Y (Microfluidics Corporation, Newton, MA) which was submerged in an ice bath to prevent overheating and possible degradation of the sample. The final emulsion was collected after five passes through the machine at 5,000 psi and stored at ambient temperature until further use. Samples intended for analysis via gas chromatography were immediately stored in 40 mL glass vials with septa screw tops while emulsion samples intended for other analyses were stored in 4 mL aliquots in sealed glass test tubes to prevent evaporation.

3.3.4 Particle Size and Charge Measurements

Particle size of the stock solutions, prior to any oil additions or emulsification, was characterized by a compact light scattering goniometer (ALV CGS-3 Compact Goniometer System, ALV, Langen, Germany). Solutions of particles were placed in a round, glass culture tube (10mm diameter) and inserted into the path of a helium neon (HeNe) laser at a wavelength of 632.8 nm. Intensity of scattered light was monitored at a 90^{O} angle with ALV High Q.E. avalanche photodiode (APD) dual detectors in pseudo-cross correlation mode and a 50:50 fiber-optical beam splitter. Cumulant or CONTIN algorithms, included in the light scattering software (ALV, etc.), were chosen on the basis of minimized fit error and used to calculate particle radius and polydispersity index (PDI) values.

A Malvern Zetasizer Nano was used to obtain ζ potential values of both stock particle solutions and final emulsions. Zeta potential values are used as an indication of the electrical potential of the particle or droplet at the slipping plane and can indicate whether or not they will be attracted or repelled from one another in solution[76]. Values greater than +30mV or less than -30mV indicate relatively high zeta potentials, meaning that the particles and/or droplets should stay suspended in solution and resist aggregation. Samples were diluted by 4x with appropriate buffer and filled in a clear, disposable, folded capillary cell (Malvern, model DTS 1060C). The Henry equation using the Smoluchowski approximation was used to predict the zeta-potential based on the average electrophoretic mobility of the particles in an alternating electrical current with the buffer viscosity taken as the continuous phase viscosity. At least 10-20 measurements were

performed for each individual sample. Additionally, automatic attenuation settings and voltage selections were employed for each sample.

3.3.5 Atomic Force Microscopy

To obtain further information on particle morphology, atomic force microscopy (AFM) was performed under ambient conditions using a MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA) in intermittent contact mode and equipped with a silicon probe with aluminum reflex coating of force constant 5 N/m (Ted Pella Inc., Redding, CA). To prepare the slides for imaging, 20 µL of 1:10 diluted sample solution was placed on a freshly cleaved mica disk (Ted Pella Inc., Redding, CA) and allowed to sit for three minutes to allow for deposition until the excess material was rinsed with pH-adjusted ultrapure water. Slides were dried with air and stored in a dessicator until analyzed. Scan sizes were done in a 1µm x 1µm range and performed on several areas of the slide to gain a representative image of the sample.

3.3.6 Drop Shape Analysis

To demonstrate the interfacial activity of the heat-treated particles, surface tension measurements were performed using the inverted pendant drop method on a Rame-Hart model 250 standard goniometer (Succasunna, NJ). This involved filling an optical, glass cuvette (Starna Cells Inc, Atascadero, CA) with 45 mL of test solution containing either unheated particles, heat-treated particles, dialyzed particles or buffer as a control. An inverted, 22 gauge, stainless steel needle (Rame-Hart Instrument Co, Succasunna, NJ) was then lowered into the solution until in the camera range. Corn oil was dispensed from

the needle using an automated dispenser until the droplet shape was distorted by gravity but stable enough to withstand the duration of measurement. An isolation table was used to prevent any disturbances from building vibrations or other equipment. Dynamic interfacial tension (σ) values were immediately collected at 5 second intervals over a time span of 90 minutes using the Rame Hart DROPimage Advanced Software. The calculated values of surface tension were converted to surface pressure using formula 4.

$$\sigma - \sigma_o = \Pi \quad (4)$$

Where σ is the interfacial tension as a function of time as the surfactant travels to the interface, σ_o is the interfacial tensions at time zero when no surfactant is present and Π is the calculated surface pressure. All units are mN/m.

These experiments were repeated in duplicate to ensure repeatability.

3.3.7 Accelerated Creaming Analyses

During the seven day study of emulsion stability, no clear separation of serum and cream layers was noted except for a fine ring of cream at the top of the storage test tube. In order to more clearly and rapidly see the resistance to creaming, accelerated creaming experiments were performed using an Eppendorf centrifuge 5415 D (Eppendorf International, Hamburg, Germany). 1 mL of freshly-made emulsion was placed into plastic 2mL centrifuge vials and tightly capped. Samples were then centrifuged at 1000, 2000, 3000, 4000, 5000, 6000, 7000, 9000, 10,000, and 13,000 rpm for 10 minutes intervals and lastly, at 13,000 rpm for 20 minutes. Once complete, all sample vials were photographed and visually compared for appearance of creamed layer, sedimentation and

clarity of serum layer. Experiments were repeated in triplicate alongside each emulsion trial with representative images shown.

3.3.8 Emulsion Drop Size and Charge Measurements

Emulsion droplet size was characterized by static light scattering using a Mastersizer 2000E (Malvern, Worcestershire, UK) to obtain both $d_{4,3}$ and $d_{3,2}$ values, as well as particle size distributions. Emulsion samples were added dropwise into 10 mM acetate buffer with a disposable plastic pipet until obscuration was between 5% and 15% (approximate dilution of 1:500). Pump speed was set to 2,000 rpm. Laser light (632 nm) scattered by the sample was collected with 45 detectors. Particle sizes were extracted from the static light scattering intensities as a function of angle by fitting to an onboard library using Mie and Fraunhofer scattering theories. The refractive index (RI) for corn oil was set to 1.466 while the dispersant water RI was 1.330 and the limonene with 10% corn oil was 1.4714. Experiments involving solely corn oil were performed in triplicate and are reported as an average +/- standard deviation while those involving limonene with 10% corn oil were performed in duplicate.

3.3.9 Headspace Gas Chromatography

Headspace gas chromatography (GC) methods, based upon the work of Mirhosseini and others[107], were used to compare the encapsulation efficiency of different emulsion trials by determining the concentration of limonene in the gaseous phase above liquid dispersions. Upon emulsification, 10 mL of emulsion were immediately stored in 40 mL borosilicate glass vials with 0.125“ silicone/PTFE septum in a white

polypropylene cap (Thermo Scientific, Waltham, MA). 50 µL of headspace air was sampled using a Pressure-Lok precision analytical syringe (VICI Precision Sampling, Inc., Baton Rouge, LA) after the emulsions had been allowed to fully equilibrate for 24 hours and fresh samples were then sampled again at day 7 to detect any changes in release over storage. Headspace samples were manually injected into the GC port with split mode conditions (10:1). The GC was equipped with a DB-Wax column (Agilent Technologies, Santa Clara, CA, 30m length, 0.25mm internal diameter, and 0.25µm film thickness) and a flame ionization detector (FID) at 250^{O}C. Oven temperature was programmed at a rate of 15 ^{O}C/min and the carrier gas was helium with flow rate of 2.4 mL/min. Samples were prepared in duplicate and results are reported as an average +/- standard deviation of the peak area of limonene. 50 µL of headspace air over 5 mL of pure, R-(+)-limonene analytical standard (Sigma Aldrich, St. Louis, MO) were injected prior to each run to obtain the correct retention time of our analyte. A standard curve of limonene headspace concentration was constructed using separate vials containing 10 mL of pure, R-(+)-limonene. Headspace sample injections of 10, 30 and 50 µL were performed using the same needle to avoid any discrepancies. A control emulsion of 1% (wt/wt) 90:10 limonene:corn oil in water, 1% (wt/wt) 90:10 limonene:corn oil in 0.5% Tween 20 and also an emulsion containing 0.05% Tween 20 and 1% 90:10 limonene:corn oil were used for comparison of headspace volatiles.

3.4 Results and Discussion

The application of heated biopolymer particles as Pickering-type stabilizers for o/w emulsions was studied. Investigations into the use of heated microgels and subsequently,

complexes, to stabilize corn oil interfaces served as the basis for the work involving the encapsulation of the volatile flavor oil, limonene.

3.4.1 Microgel Particle Characterization

Biopolymer particles composed of only heated β-lactoglobulin (microgels) at pH 5.8 were initially characterized prior to application in Pickering-type stabilized emulsions. These experiments included imaging with atomic force microscopy to obtain morphology and particle size estimations, as well as dynamic light scattering to further support AFM scans, and ζ-potential measurements to gain information about colloidal stability.

The AFM images indicated spherical, globular morphology for the 0.25% heated microgel particles (Figure 3.1.A) which is in concurrence with previously published research[13]. Samples were also collected after subjecting the stock solution to the same homogenization procedures as the emulsions in order to ensure that the particles were physically stable throughout processing (Figure 3.1.B). The scales on Figure 3.1 A and B indicate roughly 15-20 nm in height. Due to the drying effects that occur when plating the sample slides, we can assume that the microgels shrink to roughly 60% of their original size meaning that the microgels are approximately 25 to 33 nm when fully hydrated in solution. From these scans, we saw that the heated microgel particles were stable throughout the homogenization procedure and were not susceptible to degradation. One important issue to note is that a wide range of particle sizes were seen throughout AFM scans, including some larger particles around 1 micron and also clumps of aggregates which may or may not be artifacts of drying the sample slides.

This wide range of particle sizes was also seen with the dynamic light scattering data. Two populations were noticed within the heated microgel solutions. The first peak which made up roughly 40% of peak weight was centered on a radius of 18.35 nm while the second peak, making up the remaining 60% of the microgel population was centered around a radius of 153.3 nm. This peak was quite broad however, ranging from 55 to 235 nm in radius, indicating that the particle sizes are not uniform. Previous research on similar β-lactoglobulin microgels had found that pH 5.8 heated microgels produced particles with a hydrodynamic radius of 216nm and fairly narrow particle size distribution (PSD). One reason that our PSD could have been larger may have been due to differing protein purities or heating rates.

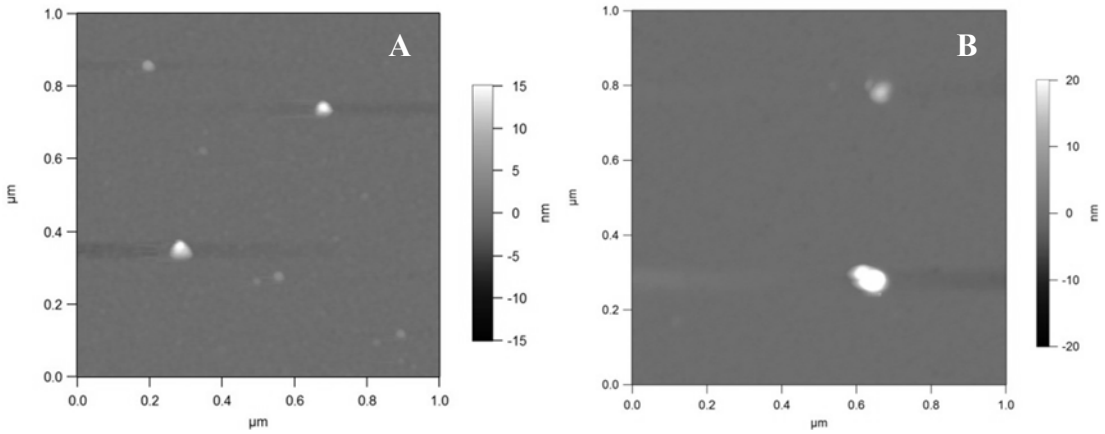

Figure 3.1 AFM height image (1µm x 1µm) of 0.25% heated microgels at pH 5.8 before (A) and after (B) homogenization procedures.

Surface charge of the 0.25% heated microgels, as measured by ζ-potential, was found to be around -28.95 ± 1.3 mV when measured at pH 5.8. As mentioned in previous sections, ζ-potential values less than -30 mV or greater than +30 mV are thought to have sufficient charge to maintain stability through electrostatic repulsion. As the heated

microgels had suitably high ζ-potential values, they have sufficient particle-to-particle repulsions to prevent aggregation or flocculation and therefore, remain stable in solution.

3.4.2 Corn Oil Emulsions Stabilized with Microgels

After characterizing the initial biopolymer particles, the microgel solutions were homogenized with 1 % (wt/wt) corn oil at different concentrations in order to see the effects of different droplet coverage levels. In Figure 3.2, a photograph of the microgel emulsions after one day of storage verify the production of a white, opaque emulsion throughout all three concentrations in both the heated and unheated particle systems.

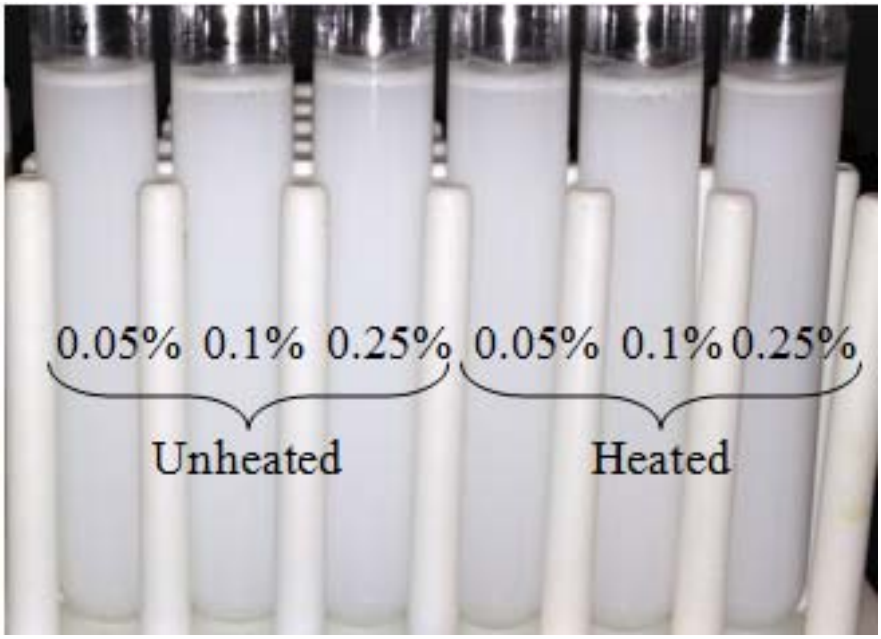

Figure 3.2 Photograph of 1% corn oil emulsions, made with either heated or unheated microgels, on day one of storage

The lack of a clear, serum layer separating from the stable, turbid emulsion is one sign of emulsion stability to gravitational separation. However, all emulsions produced

visible evidence of a thin, creamed layer ringing the top of the sample test tube which was attributed to emulsion instability within a small volume fraction. According to Stokes Law (Equation 5), larger particles tend to cream faster than smaller particles due to differences in diameter that increase their separation. Therefore, we can assume that the white ring consisted of the larger corn oil droplets that cream quickly while the smaller, stable corn oil droplets remained in solution.

$$v = (2r^2 g\Delta\rho) / (9\eta) \quad (5)$$

Where v is the velocity of separation, r is the radius of the droplet, g is the acceleration due to gravity, $\Delta\rho$ is the difference in densities between the dispersed and continuous phases, and η is the viscosity of the aqueous phase.

The oil droplets accelerate upwards, because the oil phase is less dense than the aqueous phases surrounding it and will continue until the buoyant force upward is equal to the drag force keeping it in place. No appreciable differences were noted in visual appearances at the end of seven days and no serum layer was observed, indicating that the initial creamed ring that formed within the first day was likely the only population of larger, unstable droplets. These droplets could be unstable due to inadequate coverage of the oil interface by particles, and therefore, were unstable to flocculation or coalescence without sufficient repulsive interactions, such as steric hindrance.

Figure 3.3 Centrifugation creaming index measurements of 1% corn oil emulsions stabilized with 0.05% heated microgels (top), 0.1% heated microgels (middle), and 0.25% heated microgels (bottom). All emulsions were analyzed on day zero and are in order of increasing centrifugation speed.

In order to more clearly see the effects of gravitational separation, accelerated centrifugation analyses were performed by centrifuging emulsion samples at varying speeds. The emulsions stabilized with only 0.05% heated microgels appear to separate into a serum and creamed layer the quickest, with the lower aqueous layer becoming crystal clear at lower centrifugation intervals. In contrast, the 0.25% heated microgel emulsions are able to maintain a somewhat turbid appearance at higher speeds, indicating higher stability to gravitational separation. One important observation is the appearance of a small pellet forming in the bottom of both 0.1% and 0.25% heated microgel emulsions. This is likely a portion of the heated particles which are not inserted into the

oil interface and precipitate out during centrifugation. No pellet was observed with the 0.05% heated microgel-stabilized emulsion indicating that most of the microgel must have successfully stabilized the oil droplets. Accelerated creaming index photos of the unheated microgel-stabilized emulsions are shown in Appendix Figure A.1. Very similar trends are seen with the exception of the pellets, which are not present in the unheated systems. This is an indication that all of unheated microgels across all three concentrations are able to fully stabilize the oil interfaces.

Figure 3.4 demonstrates the effects of different levels of heated and unheated microgel particle coverage on the mean droplet diameter of the 1% corn oil emulsions as represented by $d_{3,2}$ values.. At day zero, both the unheated and heated 0.05% microgel emulsions had the two highest $d_{3,2}$ drop sizes at around 383 nm and 423 nm, respectively. The smallest $d_{3,2}$ drop sizes was found with the 0.25% microgel emulsions in which both the heated and unheated particles were able to form droplets around 310 nm in diameter.

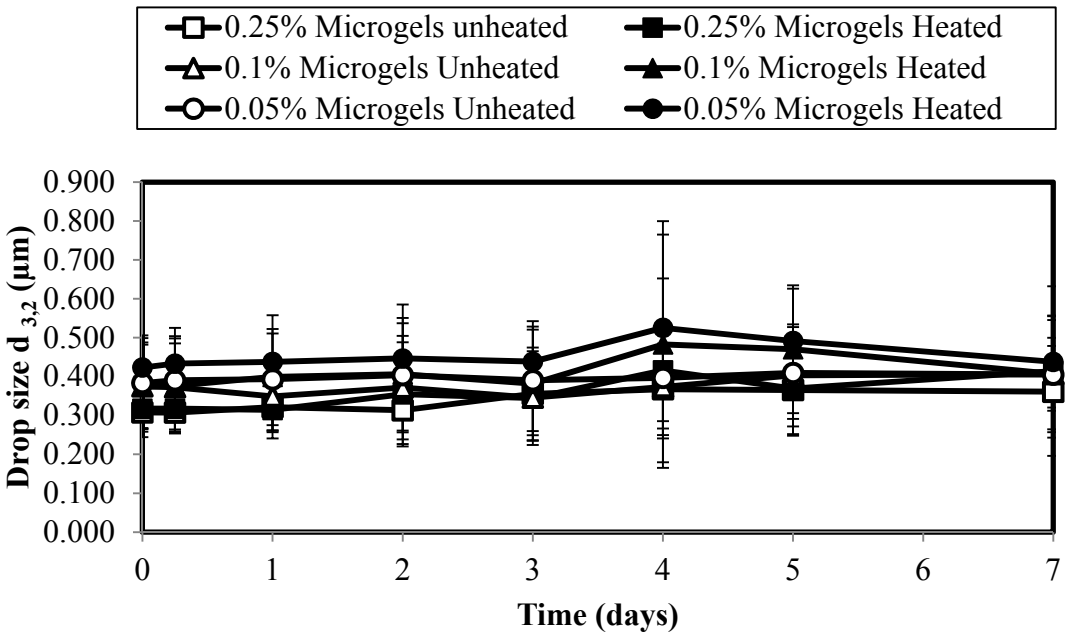

Figure 3.4 Surface-weighted droplet mean diameter ($d_{3,2}$) over seven days for both heated and unheated microgel emulsions at concentrations of 0.05%, 0.1% and 0.25% (wt/wt) with 1% corn oil (wt/wt). Error bars indicate standard deviation, n=3.

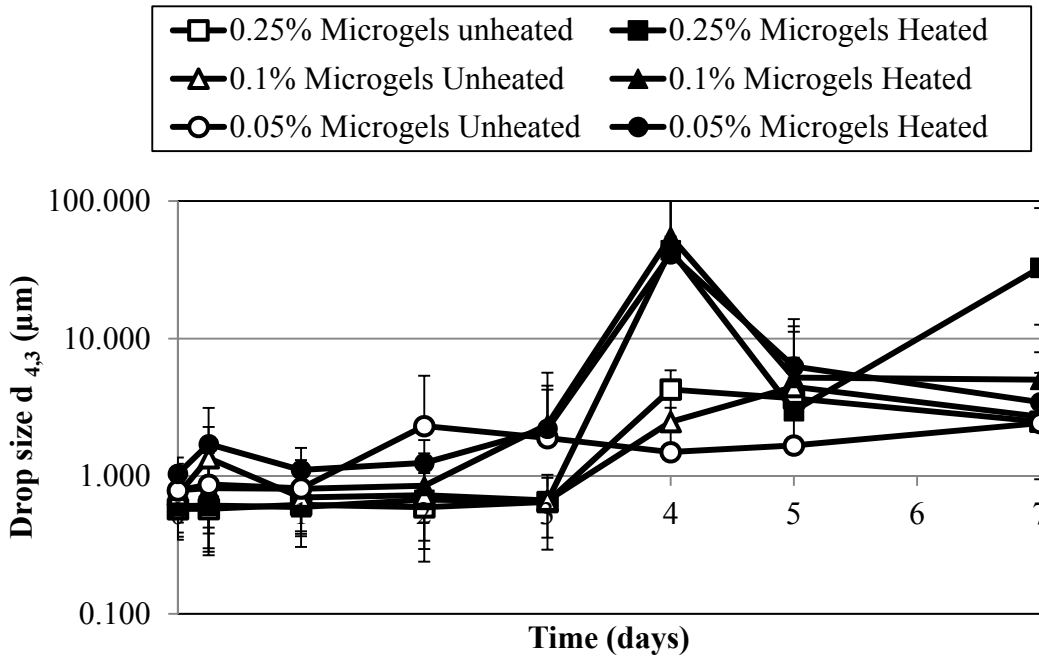

Figure 3.5 Volume-weighted droplet mean diameter ($d_{4,3}$) in log scale over seven days for both heated and unheated microgel emulsions at concentrations of 0.05%, 0.1% and 0.25% (wt/wt) with 1% corn oil (wt/wt). Error bars indicate standard deviation, n=3.

The 0.1% microgel emulsions for both heated and unheated particles fell somewhere in between these ranges. As we move throughout the seven day storage, the droplet diameters appeared to be rather stable with a slight increase in size occurring on day four but leveling out towards day seven.

In comparison, when looking at the $d_{4,3}$ droplet sizes, we noticed that the droplet mean diameters are significantly larger (Figure 3.5). This was to be expected as the $d_{4,3}$ representation of the mean droplet diameter tends to emphasize the larger population. Once again, the 0.05% levels of microgels tended to have the largest diameters (approximately 0.8 to 1 μm) as compared to the 0.25% microgel emulsions that had $d_{4,3}$ values around 600 nm on day zero. Figure 3.5 clearly demonstrates that as the particle concentration increased from 0.05% up to 0.25%, the droplet diameter began to decrease. This effect was more pronounced with the heated microgels than with the unheated microgels because the unheated particles were likely more flexible and the protein chains can uncoil at the interface thus covering more oil surface area with a lower particle concentration. Additionally, the unheated protein is much smaller in size than the heated microgels implying that at the same weight concentration, there exists a vastly greater number concentration of the unheated proteins. With 1% corn oil, the 0.05% microgels were likely unable to provide complete surface coverage of all the droplets resulting in some droplets that had exposed patches of oil. When droplets collide, they were more prone to coalescence because there was not a sufficient barrier to repel one another. Droplets will continue to coalesce until the surface coverage is sufficient enough to prevent further growth. When observing the 0.25% microgel emulsions, we saw that the droplet size was relatively stable during the initial three days, meaning that no significant

coalescence was occurring. However, from day four to seven, a significant increase in droplet diameter was observed with large variability seen between all microgel concentrations.

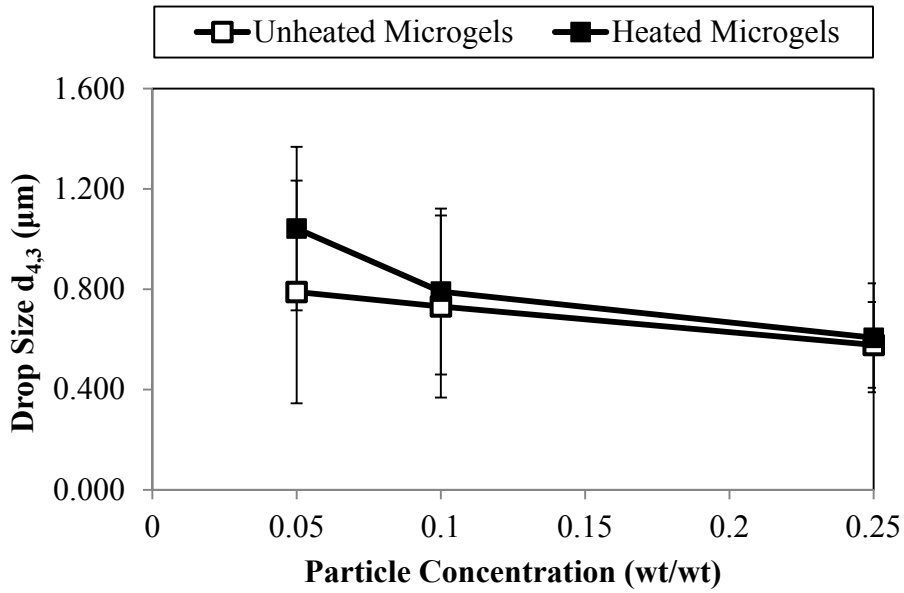

Figure 3.6 Volume-weighted mean droplet diameter after one day as a function of particle concentration for both unheated and heated microgels at pH 5.8 with 1% corn oil (wt/wt). Error bars indicate standard deviation, n=3.

When comparing between the heated and unheated microgels, the heated particle systems tended to be slightly larger in size, possibly due to the rigidity of the protein particle which doesn't unfold at the interface. Instability at the end of the seven days was still noted with the unheated particle systems however. Interestingly, even though the droplet sizes from light scattering indicated instability, the visual observations of the emulsion samples never demonstrated complete phase separation into the creamed layer and serum layer, as in several other Pickering works[7,70].

In other attempts to create stable emulsions with the heated microgels, adjustments to the ionic strength were made with additions of a concentrated sodium chloride (NaCl) solution. In theory, the added salt should screen the highly negative charges on the particles from one another, thus decreasing their electrostatic repulsion between particles. This screening effect should therefore aid the particles in adsorbing to the oil interface because there is less repulsion to inhibit them. In Appendix Figure A.2, A.3 and A.4, the droplet diameters clearly indicated that this was not successful and actually destabilized the system as evidenced by the larger droplet diameter values and visible serum layer separation.

Nevertheless, due to the high standard deviations and droplet diameters seen at the latter end of storage, these microgel particles were deemed unsuitable for further development as stabilizing agents of oil-water interfaces.

3.4.3 Complex Particle Characterization

It was decided to investigate solid particle-stabilized emulsions using a different system composed of heated β-lactoglobulin and pectin soluble complexes at pH 4.75. It was hypothesized that addition of the pectin chains would lend more stability and control to the system than when compared to the microgel system.

The objective of these experiments was to characterize the biopolymer particles before use in the subsequent emulsion trials. This included AFM scans, electrophoretic mobility measurements (as represented by ζ-potential), and dynamic light scattering to reinforce particle size distributions. The ζ-potential values for the complexes were found to be -30.5 ± 2.1 mV indicating ample electrostatic repulsion between the particles in

solution so as to prevent aggregation. Figure3.7.A shows the sizes of particles are spherical and estimated to be approximately 67 nm when in solution. When comparing this to the post-homogenization complex solution shown in Fig. 3.7. B, the same spherical shapes were present with approximately the same particle size distributions. This evidence supports the theory that these particles are durable and can withstand high-pressure homogenization.

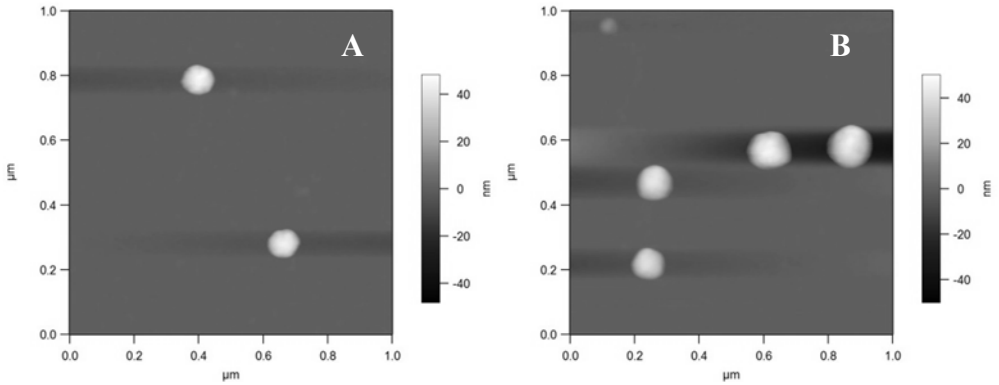

Figure 3.7 Atomic force microscopy (AFM) height images of 0.25% heated complexes at pH 4.75 before (A) and after (B) homogenization procedures.

Dynamic light scattering data indicated that the majority of the 0.25% heated complexes had a radius centered around 158.8 nm which ranged from 114.1 to 184.4 nm, indicating a small range in sizes. This is in accord with previously published literature reporting heated biopolymer particle diameters around 150 to 300 nm using a similar 2:1 β-lactoglobulin:pectin ratio[108]. These particle sizes are significantly larger than what was detected visual from the AFM scans. One reason for this discrepancy could be that dynamic light scattering measures sizes of the particles while fully hydrated in solution. It is hypothesized that the pectin lies on the outside of the heated particle[104]. Thus, the pectin chains would extend out into solution and raise the effective hydrodynamic radius.

Also detected in the heated complex solutions were smaller aggregates, likely dimers or oligomers of the protein, around 1.1 nm and 10 nm.

3.4.4 Corn Oil Emulsions Stabilized with Complexes

Emulsions composed of 1% corn oil and levels of heated or unheated complexes ranging from 0.05% to 0.25% were created in the same fashion as previously mentioned. Analyses were performed to characterize droplet size, ζ-potential and stability to accelerated creaming as well as stability to creaming in ambient environments. Figure3.8 demonstrates the corn oil emulsion stability after one day of storage. As seen with the microgel emulsions, the lower portion of the emulsion across all particle types and concentrations maintained a milky, turbid appearance that remained throughout the duration of the seven day study. Additionally, there were no apparent visual differences in turbidity between any of the samples. However, by day one, a thin ring of cream appeared on all samples indicating a fraction of droplets was unstable and susceptible to creaming. Upon briefly vortexing sample test tubes, it was observed that the ring was dispersed quite easily into emulsion. It is hypothesized that the creamed ring is composed of lightly flocculated droplets rather than larger, coalesced droplets because they were easily re-dispersed with agitation.

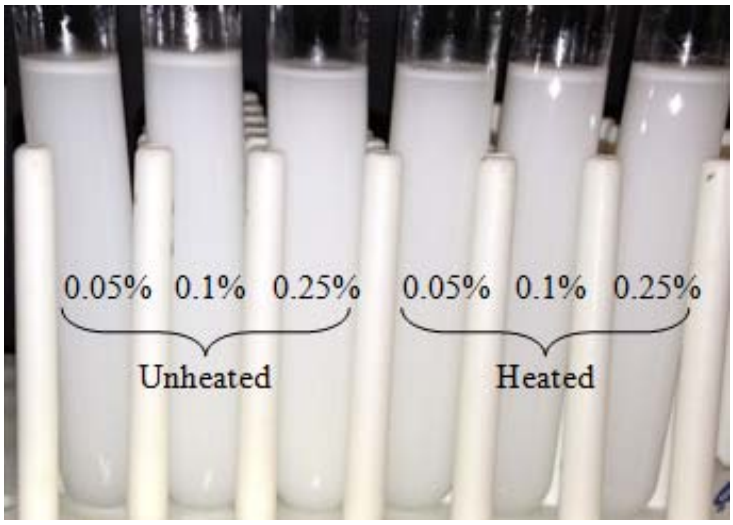

Figure 3.8 Photograph of 1% corn oil emulsions at pH 4.75, both heated and unheated complexes, on day one showing presence of creamed layer.

Figure 3.9 Centrifugation creaming index measurements of 1% corn oil emulsions stabilized with 0.05% heated complexes (top), 0.1% heated complexes (middle), and 0.25% heated complexes (bottom). All emulsions were analyzed at pH 4.75 and on day zero.

Previously, it was noted that all emulsion samples appeared to remain milky white in appearance throughout the seven days and no visible serum separation was seen. In

order to more clearly see the resistance to gravitational separation in a timely manner, accelerated creaming experiments were performed by centrifugation at increasing speeds. In Figure 3.9, 1% corn oil emulsions made with increasing amounts of heated complexes can be seen. Emulsions made at the lowest particle concentration, 0.05%, began to separate at lower centrifugation speeds, indicating the least stability to creaming. With increasing particle concentration of 0.1% and 0.25%, emulsions remained turbid at higher speeds. Also noted across all emulsion samples was the presence of the creamed layer at the top of the tube indicating that a portion of the oil droplets were unstable to the centrifugation speeds. Additionally, pellets of insoluble complexes were seen throughout all the particle concentrations. The smallest pellet was observed at 0.05% heated complexes while the largest pellet was found in the emulsions made at 0.25% heated complexes. This indicates the presence of heated complexes which were not fully adsorbed at the oil interface and were present in the aqueous phase, possibly contributing to increased viscosity. Similar trends were seen in the unheated soluble complex system with the exception of slightly smaller pellets found in the bottom of the tubes (Appendix Figure A.5). This is an indication that a higher fraction of the unheated, soluble complexes were able to adsorb to the oil interface.

Static light scattering measurements were used to determine the mean droplet diameter of each emulsion during the seven day study. In Figure 3.10, the drop size as represented by $d_{3,2}$ values are shown. Immediately from this graph we can notice that the mean droplet diameter was quite stable up to day five. At this point, the corn oil emulsion stabilized with 0.05% heated complexes showed a significant increase in surface-

weighted mean droplet diameter to approximately 3.5 µm whereas the other concentrations remained at or near their original d $_{3,2}$ mean droplet diameter values.

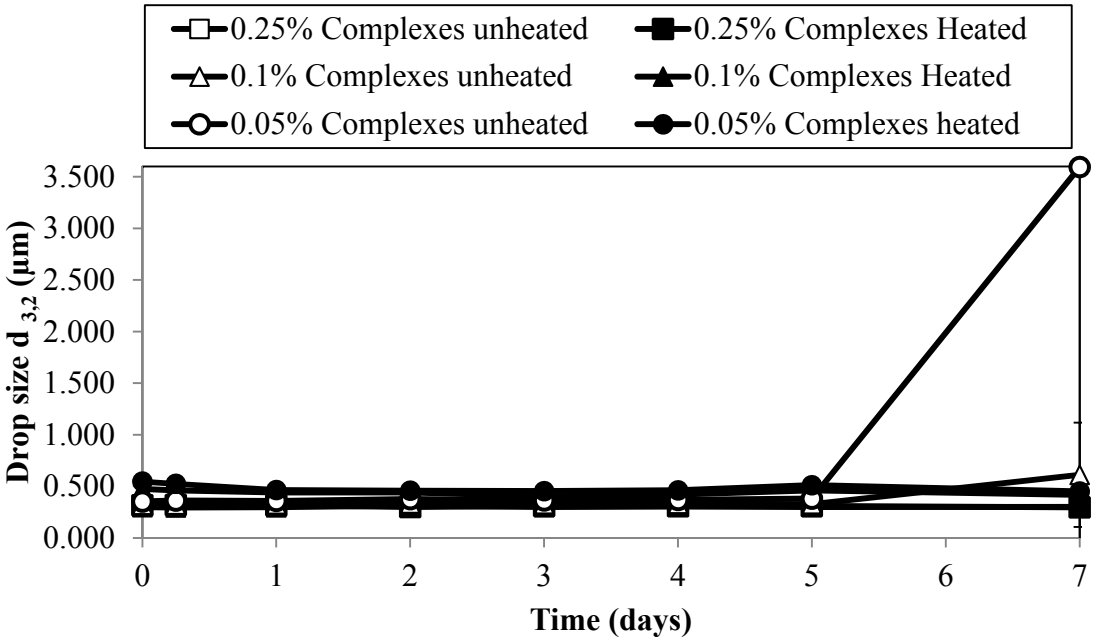

Figure 3.10 Surface-weighted droplet mean diameter (d $_{3,2}$) over seven days for both heated and unheated complex emulsions at concentrations of 0.05%, 0.1% and 0.25% (wt/wt) with 1% corn oil (wt/wt). Error bars indicate standard deviation, n=3.

In Figure 3.11, the emulsion d $_{4,3}$ droplet mean diameters are shown. This graph indicates that the emulsion droplets made using 0.25% complexes, both heated and unheated, were the smallest and most stable over time. The 0.05% heated complex emulsions started out with the highest initial drop size at approximately 550 nm while the 0.25% heated complex emulsions were around 310 nm at day zero. The drop size diameters on day zero as affected by particle type and concentration are depicted in Figure 3.12. The corn oil emulsions using unheated complexes, regardless of the particle concentration, remained quite stable around 300-350 nm. In contrast, we saw a definitive decrease in day zero droplet size as the heated complex particle level increased from 0.05%

up to 0.25%. Interestingly, the unheated and heated particle at 0.25% complexes formed emulsions with quite similar droplet sizes. This was a similar trend as shown in Figure 3.6 with the microgels but the change in droplet diameter with stabilizer concentration was relatively more pronounced. At day seven, both the unheated and heated 0.05% complex emulsions as well as the 0.1% unheated complex emulsion had increased in droplet diameter indicating instability and coalescence. The smaller, stable droplet diameters seen in the 0.25% complex emulsions again indicated that even though a creamed ring is present in Figure 3.8, the flocculated, creamed ring was dispersed upon vortexing into individual droplets once again. This was also an indication that the droplet coverage at 0.25% was sufficient to prevent coalescence.

One interesting aspect of the complex emulsions as compared to the previously studied microgel emulsions was the standard deviation values seen between the two. Much greater variability was seen with the microgels whereas the complexes were much more consistent and precise. This increased predictability in size would be advantageous when formulating emulsions in the future.

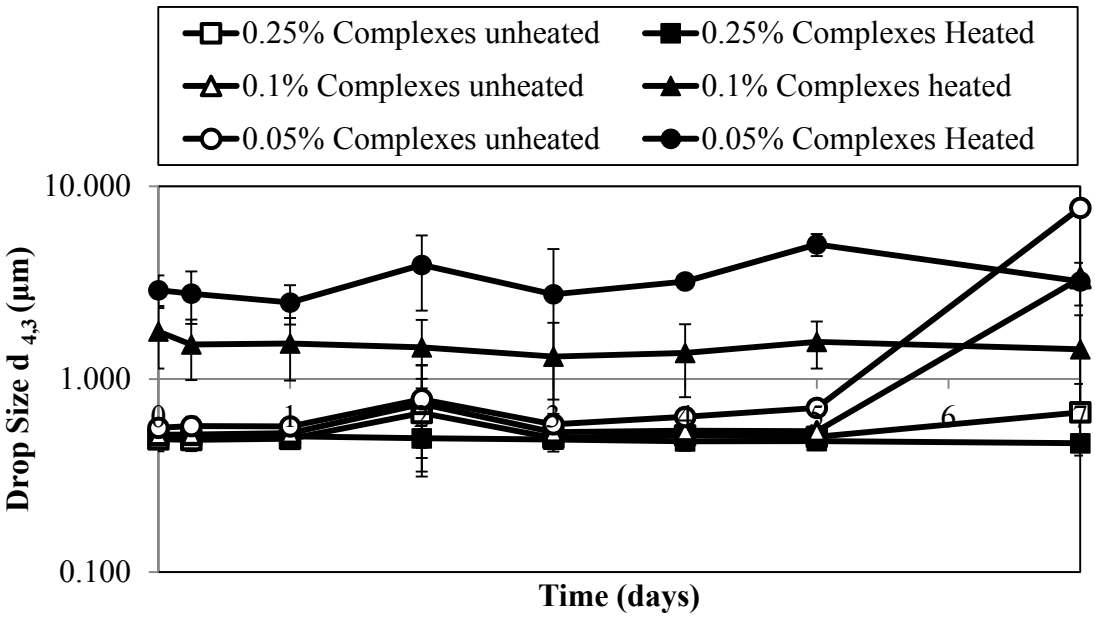

Figure 3.11 Volume-weighted droplet mean diameter ($d_{4,3}$) in log scale over seven days for both heated and unheated complex emulsions at concentrations of 0.05%, 0.1% and 0.25% (wt/wt) with 1% corn oil (wt/wt). Error bars indicate standard deviation, n=3.

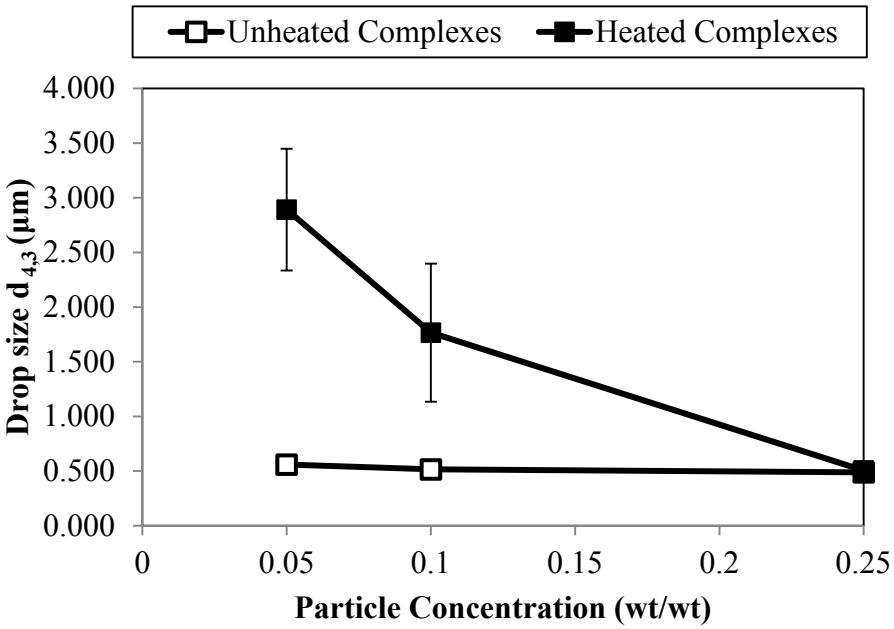

Figure 3.12 Volume-weighted mean droplet diameter as a function of particle concentration for both unheated and heated complexes at pH 4.75 with 1% corn oil (wt/wt). Error bars indicate the standard deviation in particle sizes, n=3.

Similarly to the microgel trials, the addition of a concentrated NaCl solution to the complex-stabilized emulsion was investigated. The theory behind this experiment was that the electrostatic repulsion between particles would be diminished by charge screening from the salts, so that in turn, interfacial adsorption would be improved. Appendix Figure A.4, A.6 and A.7 showed that both $d_{4,3}$ droplet mean diameter values and visual observation indicated that the added salt destabilized the emulsion system. Therefore, it was decided to forego the salt additions.

Dialysis procedures were performed in order to remove any free protein or smaller aggregates. AFM images were collected after the seven day dialysis treatment to observe the population of particles remaining and to ensure the same spherical particle morphology was present (Figure 3.13). A stock solution of 0.25% heated complexes was used to generate the standard curve via UV-VIS turbidity measurements. Using this standard curve (Appendix Figure A.8), it was found that the dialyzed complex solution contained roughly 0.23% protein as compared to the 0.25% at the beginning. This indicated that the majority of the β-lactoglobulin and pectin that was initially present was converted into the complexes rather than remaining free in solution.

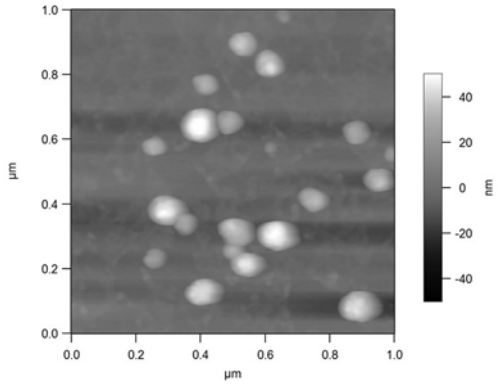

Figure 3.13 AFM height image (1 µm x 1µm) of 0.25% heated complexes at pH 4.75 after one week of dialysis treatment.

When comparing the $d_{3,2}$ droplet diameters of the 0.25% heated complexes and the dialyzed heated complex emulsions in Figure 3.14, we saw that the droplet sizes were quite similar at the beginning of the study with both emulsions averaging 320 nm for the mean droplet diameter. The mean droplet diameter for the dialyzed, heated complex-stabilized emulsion maintained a fairly steady droplet diameter throughout the seven day storage. However, the undialyzed, heated complex-stabilized emulsion appeared to exhibit a shift in mean droplet diameter from the original 320nm down to approximately 300 nm on day seven. Although this was not an obviously substantial difference, more information can be elucidated from the graph of the volume-weighted surface diameters in Figure 3.15.

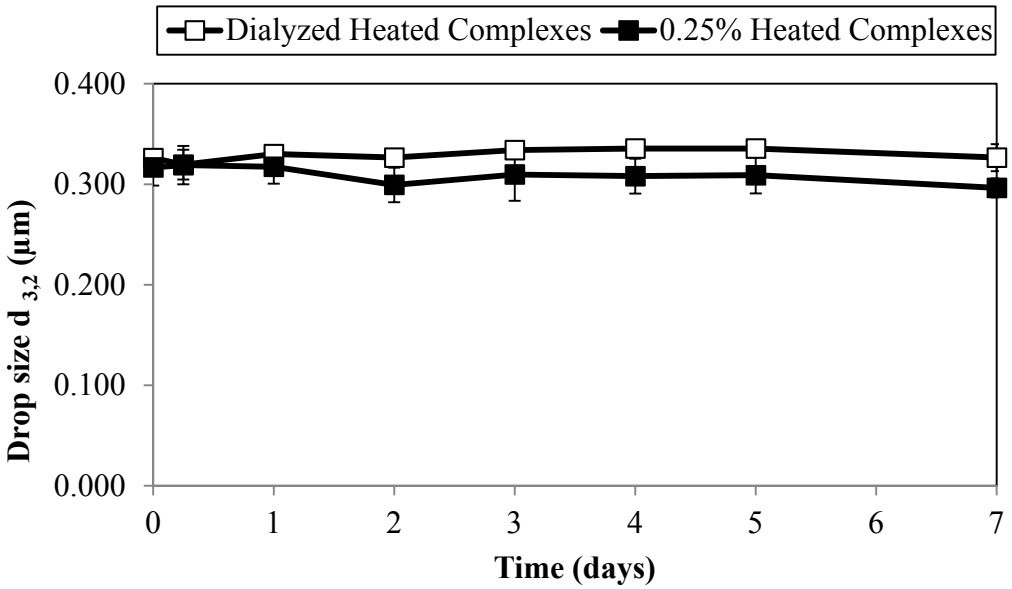

Figure 3.14 Surface-weighted droplet mean diameters ($d_{3,2}$) over seven days made with both heated complex particles and dialyzed, heated complex particles. Error bars indicate standard deviation, n=2.

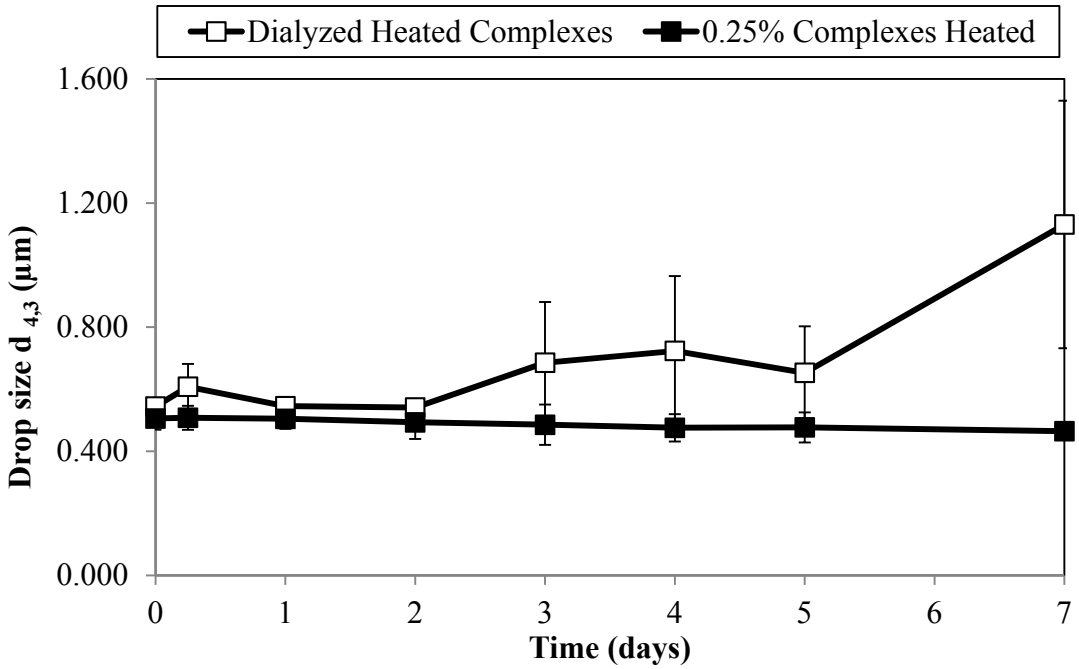

Figure 3.15 Volume-weighted droplet mean diameters ($d_{4,3}$) over seven days made with both heated complex particles and dialyzed, heated complex particles. Error bars indicate standard deviation, n=2.

Although the emulsion droplet diameters appeared stable over seven days when observing the $d_{3,2}$ values, Figure 3.15 indicates substantial droplet growth of the mean $d_{4,3}$ values for the dialyzed, heated complex emulsions. On day seven, the dialyzed, heated complex emulsions had grown to over 1 µm in diameter whereas the undialyzed, heated complex emulsions were consistently around 460-500 nm in diameter from day zero throughout day seven. This suggested that the lack of the free protein or small aggregates, which were removed during the dialysis procedures, played an important role in stabilizing the corn oil-water interface and preventing coalescence. Several studies have investigated the role of added surfactants with nanoparticles at an oil-water interface in order to enhance the particle attachment[69,109]. Specifically, Vashisth and others found that upon increasing the amount of sodium dodecyl sulfate in a silica nanoparticle stabilized

oil emulsion, smaller droplet sizes were seen[74]. They hypothesized that the small surfactant molecules, SDS in their study, adsorbed to the interface quickly to reduce interfacial tension thus allowing the oil to be readily fragmented into smaller droplets which were then stabilized by the silica nanoparticles. At levels of surfactant above the critical micelle concentration (CMC) however, displacement of the silica nanoparticles occurred. In our experiment, the level of free protein removed was estimated to be approximately 0.02% as indicated by the difference in absorption values before and after dialysis. It is possible that this small amount of free protein is not enough to cause particle displacement at the interface, rather that it fits in between the nanoparticle packing structure helping to stabilize the emulsions and decrease surface tension. In the case of the dialyzed, heated complex emulsions, this lack of the smaller, free protein was enough to cause instability within the system because there was no surfactant to initially decrease the interfacial tension at shorter times. Further experimentation would be necessary to prove this hypothesis but it would be interesting to analyze the interfacial tension of dialyzed, heated complex emulsions with increasing levels of free protein to see the effects on droplet stability and particle attachment.

3.4.5 Complex Emulsions with 90:10 Limonene: Corn Oil

The objectives of these experiments were to apply the heated complexes as particle stabilizers for a volatile flavor oil and to investigate if the heated particles could inhibit the rate of volatile flavor loss. Based upon the results from the initial corn oil trials, it was decided to use the 0.25% heated complexes for this application due to the stability imparted at that concentration for a 1% oil-phase fraction and decreased variability seen

with the heated complexes. It was decided to also include a fraction of the poorly soluble corn oil along with the limonene flavor oil to inhibit against potential Ostwald ripening, as was demonstrated with modified starch-stabilized emulsions with a combination of corn oil and orange oil by McClements et. al[84]. Tween 20 was chosen as the control surfactant and was used at both the 0.05% and 0.5% usage level in order to have two comparisons. The lower level of 0.05% Tween 20 should be acceptable to stabilize 1% corn oil, which corresponds to similar oil:surfactant ratios previously used to emulsify soybean oil with Tween 20[110]. The higher level of Tween 20 (0.5%) tested the effects when large excesses of surfactant were present that could accommodate micelle formation and possibly solubilization of the limonene.

In Figure 3.16, the effect of the different emulsifiers was seen after one day of storage. The 0.25% heated complex-stabilized emulsion appeared very white and opaque while the 0.05% Tween 20 stabilized emulsion was slightly less opaque but still appeared a milky white. In contrast, the 0.5% Tween 20 stabilized emulsions appeared translucent. This may be attributed to the formation of very small micelles of limonene which do not scatter light strongly and therefore, appeared to be clear. Interestingly, no creamed layer appeared in the 0.25% heated complex emulsion throughout the seven day study as was seen with the previous trials involving only corn oil. The combination of limonene with a small fraction of the bulky triglyceride, corn oil, may have inhibited the Ostwald ripening effect that was attributed to the creamed ring. A very thin, creamed ring was noted in the 0.05% Tween 20 emulsion, indicating that a small fraction of these oil droplets were unstable to gravitational separation. No creamed layer or separation was seen in the 0.5%

Tween 20-stabilized emulsion indicating that the surfactant was able to successfully stabilize the 1% limonene:corn oil that was added.

Figure 3.16 Photograph of 1% 90:10 limonene:corn oil emulsions after one day of storage made with 0.25% heated complexes at pH 4.75 (A), 0.05% Tween 20 (B) and 0.5% Tween 20 (C).

Figure 3.17 Centrifugation creaming index measurements of 1% 90:10 limonene:corn oil emulsions stabilized with 0.25% heated complexes (top), 0.05% Tween 20 (middle), and 0.5% Tween 20 (bottom).

To gain more information about the emulsion stability with the limonene-corn oil blend, centrifugation experiments were again performed at increasing speed intervals. From Figure 3.17, we can see that in the emulsions stabilized with 0.25% heated complexes, a creamed layer again separates from the turbid, lower phase indicating a small fraction of larger, unstable oil droplets. An insoluble pellet was also seen at the higher speeds indicating an excess of particles which were not attached to the oil droplet interface. This pellet was not present in either of the 0.05% or 0.5% Tween 20 emulsions. Additionally, both emulsions stabilized with Tween appeared to be somewhat clearer than the heated complex emulsion which is likely because they were initially both more transparent.

The surface-weighted mean droplet diameters are depicted in Figure 3.18.A. We saw that the 0.25% heated complex-stabilized emulsion appeared to be relatively stable throughout the seven days with initial droplet mean diameter around 305 nm. This droplet mean diameter shifted steadily down to around 240 nm at the end of the study. In contrast, there was a noticeable increase in surface-weighted mean droplet diameter in the 0.05% Tween 20- stabilized emulsion after one day indicating that droplet growth was occurring and a fraction of the oil droplets were unstable. This increase appeared to decrease after one day however, and may have been an error due to insufficient vortexing to break up the flocculated creamed ring. In the case of the 0.5% Tween 20-stabilized emulsions,, they appeared to be quite stable at the beginning of the study with mean diameter of 250 nm and shifting in population mean diameter towards 320 nm.

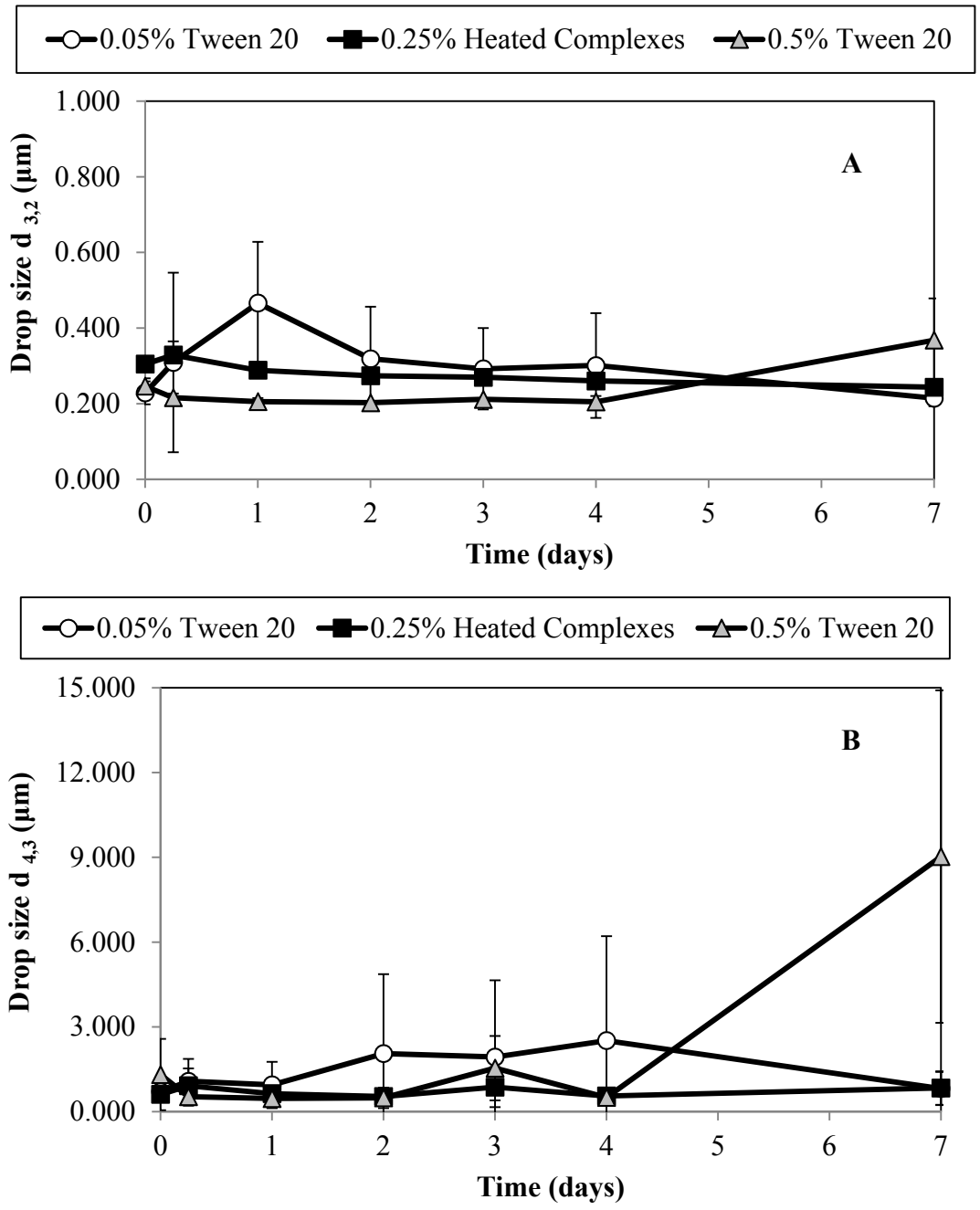

Figure 3.18 Droplet mean diameter expressed as $d_{3,2}$ (A) and $d_{4,3}$ (B) values over seven days of 1% 90:10 limonene:corn oil emulsions made with either0.05% Tween 20, 0.25% heated complexes at pH 4.75 or 0.5% Tween 20. Error bars indicate the standard deviation in particle diameter, n=3.

Larger droplet mean diameters were seen in Figure 3.18.B; however, the 0.25% heated complex-stabilized emulsion appeared to again be the most stable over seven days. Mean droplet diameters for the emulsion started around 620 nm but grew to 880 nm after seven days. As previously mentioned, no creamed ring was noted in Figure 3.14 for this 0.25% heated complex-stabilized emulsions. Even though the mean droplet diameter grew over time, the larger droplets must have been resistant to gravitational separation therefore we saw no creamed layer or serum separation. As for the 0.05% Tween 20-stabilized emulsions, much larger mean droplet diameters were seen. At time zero, the volume-weighted droplet mean diameter began at approximately 700 nm but displayed high amounts of variability as evident by the larger standard deviation bars throughout the seven days. On day seven of storage, mean droplet diameter was approximately 1.1 μm which was to be expected because of the creamed ring that appeared in Figure 3.16. Interestingly, we saw the largest mean droplet diameter value on day seven of the 0.5% Tween 20-stabilized emulsion although it never exhibited a creamed ring or had visual particulates. One reason for this could be the detection limits of the Mastersizer light scattering instrument. With this high level of surfactant, micelles were likely formed which caused problems with insufficient scattering information for the detectors.

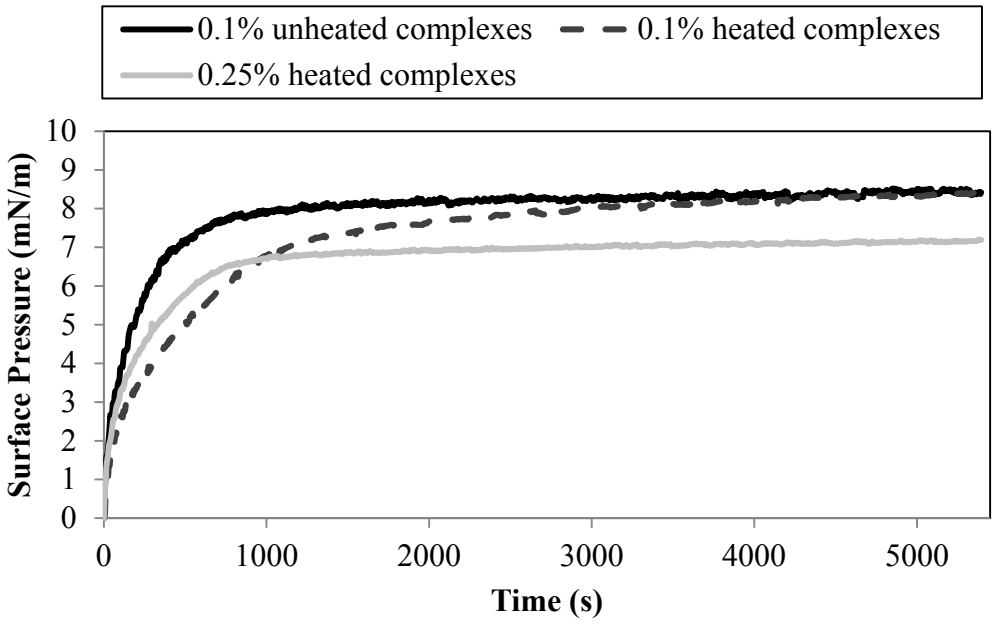

Figure 3.19 Change in surface pressure over 1.5 hours of a droplet of 90:10 limonene:corn oil in pH 4.75 complex solutions.

As demonstrated by Figure 3.19, both the unheated and heated complex particles were able to increase the surface pressure of a blend of 90:10 limonene:corn oil. An inverted pendant drop method was used to measure the effects of different solutions of complex particles over an extended period of time. The 0.1% unheated complexes were faster at increasing the immediate surface pressure of the oil droplet when compared to the heated complex particles at the same concentration. However, as time goes by we observed that the final equilibrium surface pressures were the same for both the 0.1% heated and unheated complexes. This suggested that the larger, heated complexes traveled slowly through the solution and took longer to insert themselves at the oil-water interface while the unheated complexes diffused rather quickly to increase surface pressure. We also noted that that at a higher concentration of particles, the 0.25% heated

complexes; the surface pressure was increased at a faster rate as demonstrated by the steeper slope at initial time points.

It may also be of interest that the heated particles seemed to exhibit two different slopes of adsorption. It is postulated that the first, steeper slope was the increase of surface pressure due to smaller aggregates and free proteins which diffused through solution to the interface very quickly. Bouyer et. al. found that at an oil-water interface, β-lactoglobulin was responsible for the immediate and significant decrease in interfacial tension while the secondary gum Arabic followed with much slower adsorption[111].The second, less steep slope was attributed to the adsorption of larger complexes which further increased surface pressure but took longer to diffuse to the interface due to their size. This could also help to explain the difference in emulsion stability between dialyzed and undialyzed heated complex emulsions because of the lack of free proteins and smaller aggregates to quickly decrease interfacial tension. It is interesting to note that when comparing the surface activities of individual biopolymers and the complexes consisting of two biopolymers, interfacial tension values are found to be lower at oil-water droplet interfaces than the individual components alone[111,112]

To test the ability of the heated complexes to inhibit volatilization of the limonene into the headspace, gas chromatography methods were employed to sample the headspace volatile concentrations after one day of ambient storage (Figure 3.20). Once again, 1% 90:10 limonene corn oil emulsions stabilized with Tween 20 at 0.05% or 0.5% were used as comparisons. Additionally, samples of the pure limonene oil were injected. A Tukey test was performed to test for significant differences between the peak areas obtained for each injection. Despite the presence of the heated complexes or Tween 20, no significant

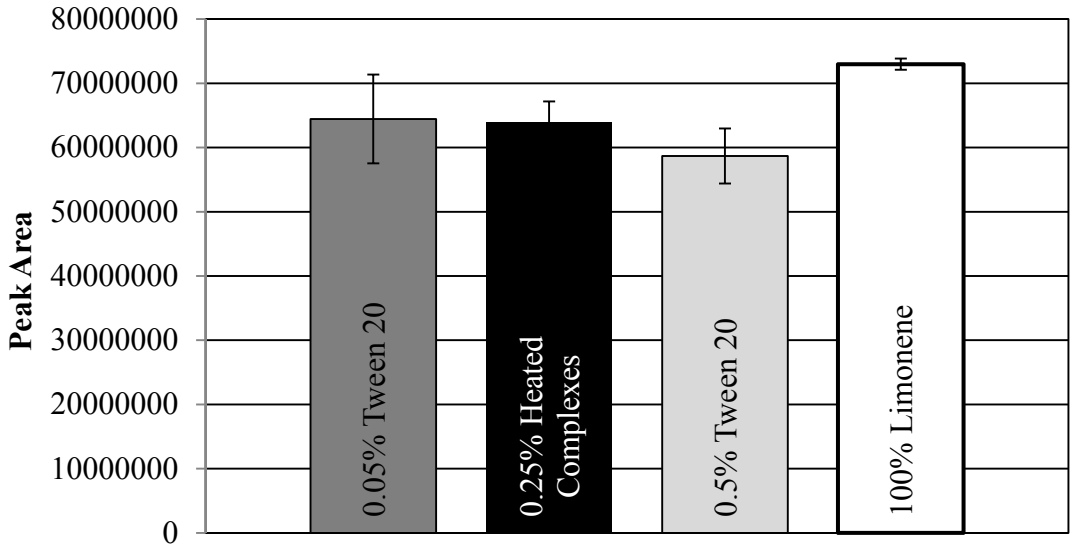

Figure 3.20 Relative headspace concentrations of limonene above 1% 90:10 limonene:corn oil emulsions containing 0.05% Tween 20, 0.25% heated complexes, and 0.5% Tween 20 as compared to the headspace volatile concentration above pure limonene. Standard deviations are indicated with error bars, n=2.

differences were found between any of the emulsion headspace volatile concentrations. The pure limonene oil was also not found to be significantly different from any of the emulsified limonene:corn oil blend. This would suggest that by the time of sampling, the emulsion had already reached thermodynamic equilibrium and the peak area values were all similar. It would be interesting in future studies to study the kinetic release of the limonene into the headspace to determine the time at which the limonene completely saturates the headspace and the rate at which it is released. It may be possible that the 0.25% heated complexes were able to slow the immediate kinetic release of limonene volatilization from the droplets but further experimentation would be required to make this claim. When comparing the evaporation rates of a limonene emulsion stabilized by either silica nanoparticles or sodium dodecyl sulfate (SDS), Binks et.al. found that the silica was able to depress evaporate rates further than the surfactant, SDS[6]. This was

performed with dynamic headspace sampling immediately after emulsion formation so samples were not allowed to equilibrate with the headspace. These methods would be useful to recreate in future experiments that look into the kinetic release of these heated complex-stabilized limonene emulsions.

3.5 Conclusion

In this study we found that heated particles made of biopolymers can act as Pickering stabilizers of an oil-water interface. Beginning with the heated microgel particles, AFM scans indicated spherical morphology with DLS data indicating a wide particle size distribution from 55 nm to 235 nm in radius. The heated microgels maintained sufficient electrostatic repulsion at -28.95 mV. When applied to a 1% corn oil emulsion, all levels of both heated and unheated microgel particle concentrations maintained a turbid, white emulsion with a thin creamed ring appearing after 24 hours. The highest concentration of 0.25% heated and unheated microgel-stabilized emulsions maintained the smallest mean droplet diameters over the seven days with $d_{4,3}$ mean droplet diameters. However, due to the large variability and increase in mean droplet diameters seen near the end of the seven day study, it was decided that the microgels were not the best candidates for Pickering emulsion stabilizers. If the microgels are further studied, it would be beneficial to further understand how to obtain more uniform particle sizes. This could potentially involve studies investigating heating rate, varying pH and ionic strength conditions, as well as other proteins such as α-lactalbumin.

The heated β-lactoglobulin/pectin complexes were consequently investigated as a viable alternative to the microgels. AFM scans again indicated uniform, spherical

morphology while DLS data revealed the majority of particle radii ranged in size from 114 nm to 184 nm. Surface charge was also adequate at -30.5 mV. When emulsified with 1% corn oil at 0.05%, 0.1% and 0.25% (wt/wt) particle concentrations, again both heated and unheated particle systems exhibited a creamed ring after one day but no separating serum layer was visible, indicating resistance to gravitational separation. The smallest $d_{4,3}$ mean droplet diameters at around 500 nm were found at the highest particle concentrations of 0.25%. As heated complex particle concentration was increased, the mean droplet diameter at time zero decreased accordingly.

Dialysis experiments to remove the free, unaggregated protein from the particle solutions resulted in a roughly 0.23% (wt/wt) complex particle solution. The dialyzed complex particles were still spherical in morphology as proven by AFM scans, but when emulsified with1% corn oil, $d_{4,3}$ mean droplet diameters were significantly larger than the undialyzed complex systems which remained stable over the seven day period. This was attributed the lack of smaller, free protein to initially stabilize the oil droplet before the larger complexes could stabilize the oil-water interface further.

The most stable set of variables, the 0.25% heated complexes, were then applied to a 1% 90:10 limonene: corn oil emulsion. No creamed ring was seen in these emulsions and turbidity was maintained throughout the seven days indicating stable emulsions resistant to gravitational separation. $D_{4,3}$ mean droplet diameters indicated slight growth from 620 nm to 880 nm but this growth must not have been substantial enough to cause visible separation or creaming. Future work should investigate if different fractions of corn oil in the limonene/corn oil blend could lead to smaller or more consistent droplet sizes instead of always using corn oil only at 10%.

Headspace gas chromatography analysis indicated that after one day, there was no significant different in limonene headspace concentrations in the 0.25% heated complex-stabilized emulsion or the emulsions stabilized by 0.05% and 0.5% Tween 20. This indicated that the emulsion had already reached thermodynamic equilibrium with the headspace so no differences were detected. However, the particles were able to demonstrate interfacial activity as seen by the increase in droplet surface pressure of the blend of 90:10 limonene: corn oil. Additionally, two different adsorption profiles were seen when comparing heated and unheated complexes. This was attributed to the quickly adsorbing, smaller, free protein and the slower adsorbing, large complexes. More work is need to conclusively understand the relationship between particle type and surface pressure, as well as the effects of free protein on the rate of surface pressure increase. Future work should investigate the effects of additional free protein as compared to the effect of solid particle concentration on the surface pressure.

It would also be beneficial to investigate the kinetic release rather than the thermodynamic headspace content of limonene from the Pickering-type emulsions in order to understand if the particles can exert an effect on the rate of diffusion under shorter time scales. Because no significant difference was noted after one day of storage, we know that all of the release has already occurred and thus are unable to differentiate between any emulsion trials in terms of inhibition of volatilization. The information gained in this research can be a useful tool in designing beverages and other food emulsion products, as well as for understanding the factors which can contribute to Pickering emulsion stability.

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APPENDIX

APPENDIX

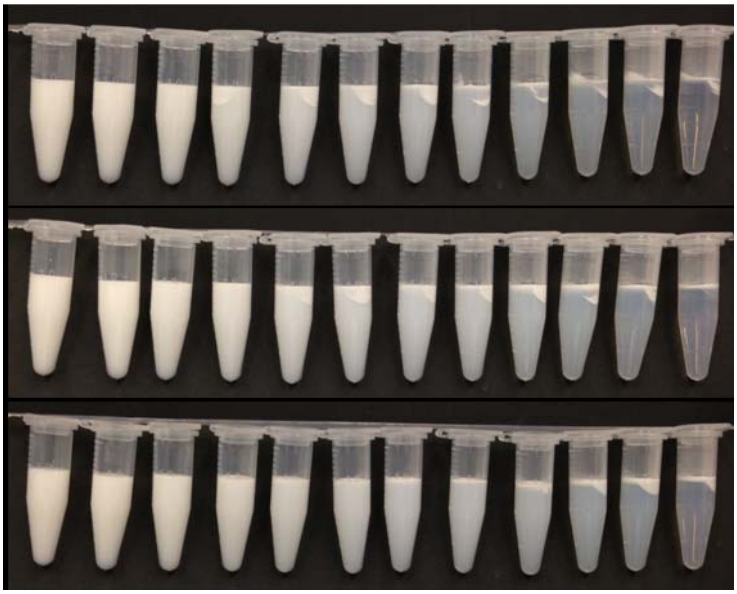

Figure A. 1 Centrifugation creaming index measurements of 1% corn oil emulsions stabilized with 0.05% unheated microgels (top), 0.1% unheated microgels (middle), and 0.25% unheated microgels (bottom). All emulsions were analyzed at pH 5.8 and on day zero.

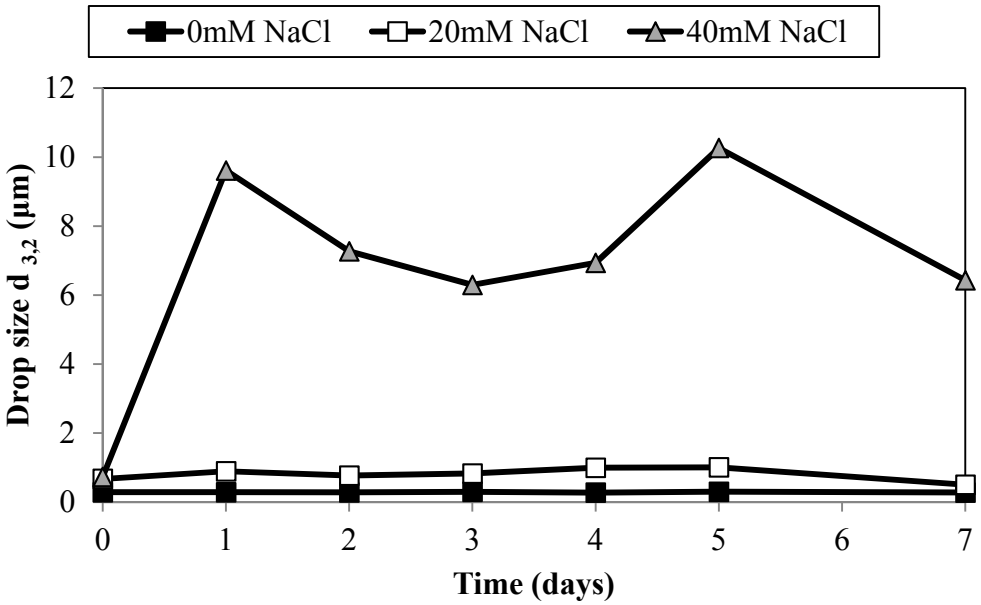

Figure A. 2 Surface-weighted mean droplet diameter ($d_{3,2}$) over seven days of 1% corn oil emulsions made with 0.25% pH 5.8 heated microgels at 0 mM NaCl, 20 mM NaCl and 40 mM NaCl.

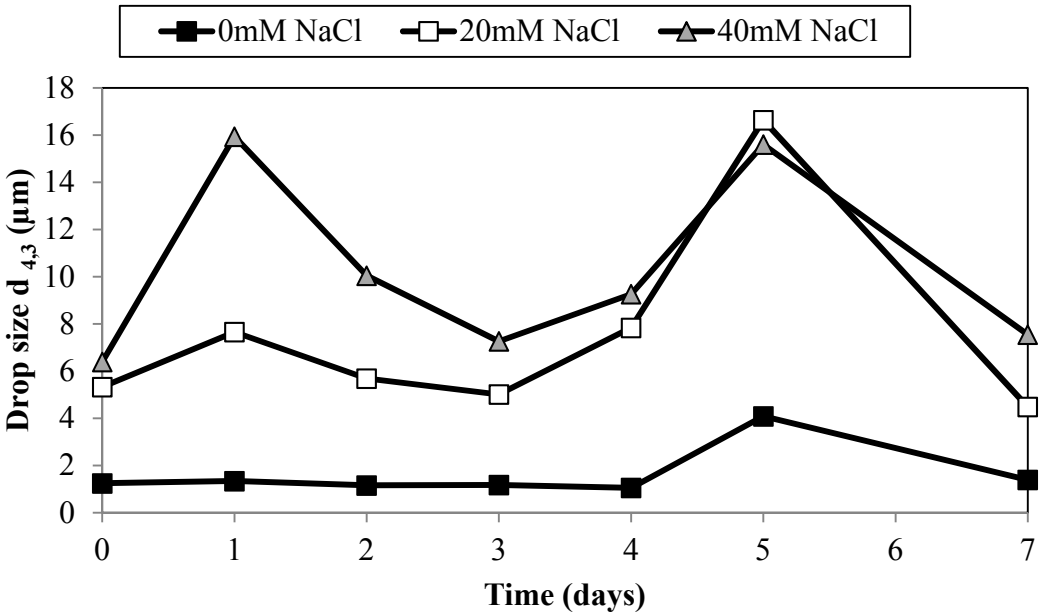

Figure A. 3 Volume-weighted mean droplet diameter ($d_{4,3}$) over ten days of 1% corn oil emulsions made with 0.25% pH 5.8 heated microgels at 0 mM NaCl, 20 mM NaCl and 40 mM NaCl.

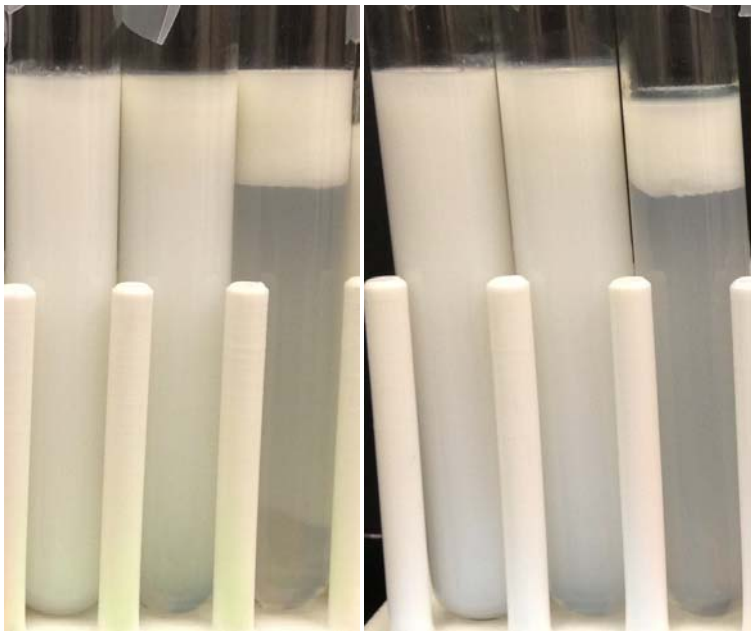

Figure A. 4 Photographs showing separation of creamed layer and serum phase of 1% corn oil emulsions made with 0.25% heated microgels (left) and 0.25% heated complexes (right) with 0 mM NaCl, 20 mM added NaCl and 40 mM added NaCl (left to right).

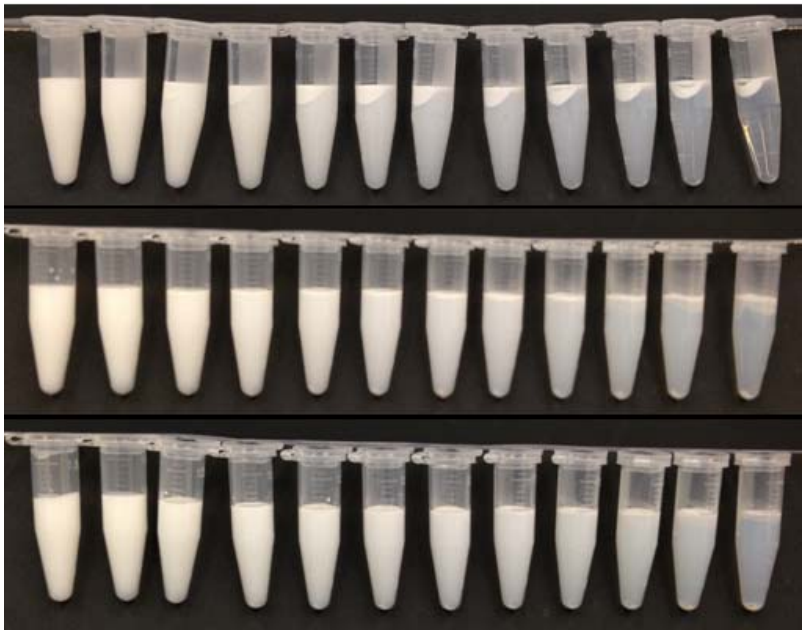

Figure A. 5 Centrifugation creaming index measurements of 1% corn oil emulsions stabilized with 0.05% unheated complexes (top), 0.1% unheated complexes (middle), and 0.25% unheated complexes (bottom). All emulsions were analyzed at pH 4.75 and on day zero.

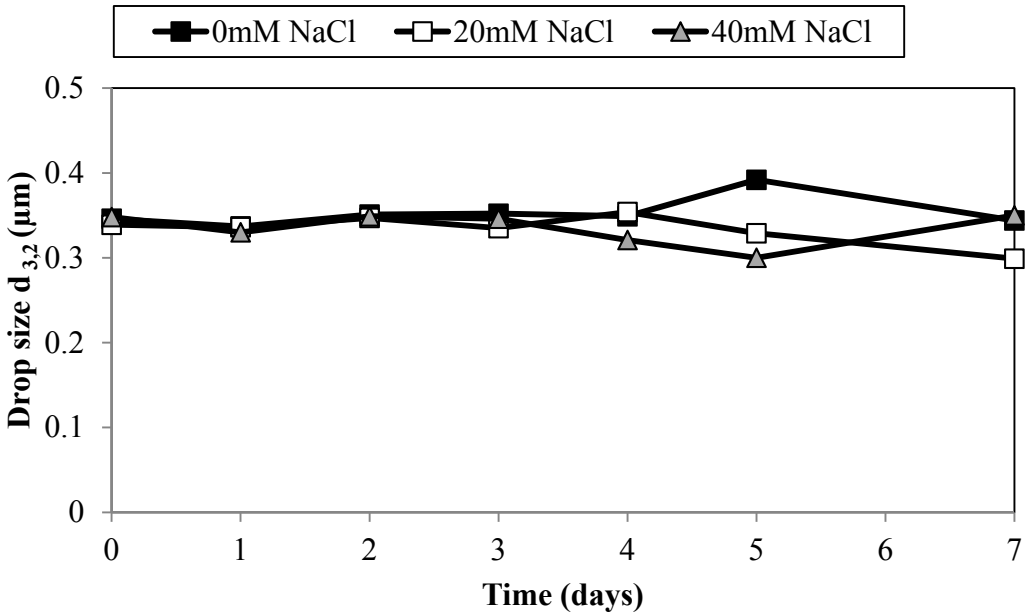

Figure A. 6 Surface-weighted mean droplet diameter ($d_{3,2}$) over seven days of 1% corn oil emulsions made with 0.25% pH 4.75 heated complexes at 0 mM NaCl, 20 mM NaCl and 40 mM NaCl.

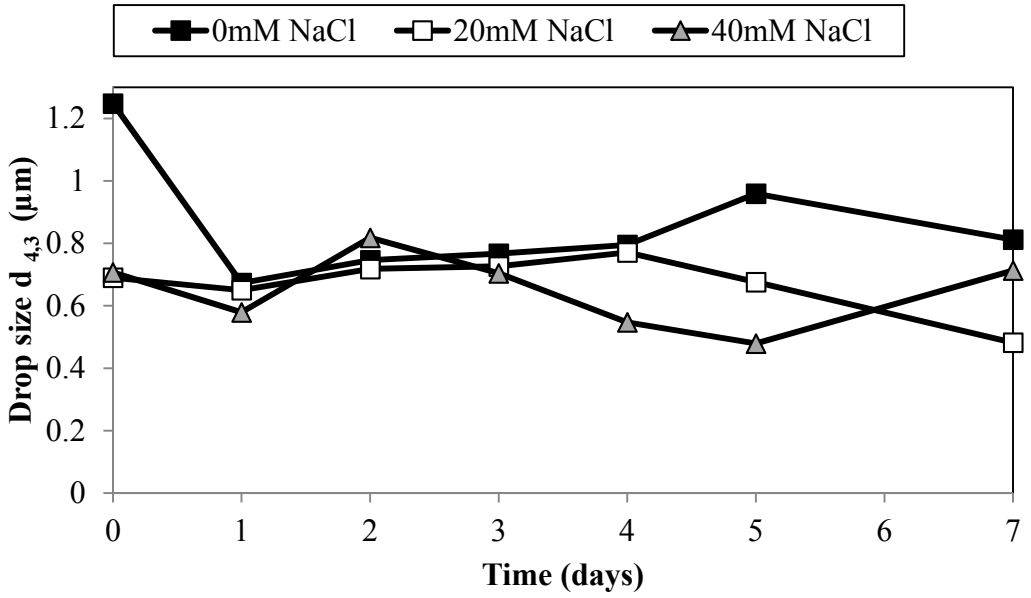

Figure A. 7 Volume-weighted mean droplet diameter ($d_{4,3}$) over ten days of 1% corn oil emulsions made with 0.25% pH 4.75 heated complexes at 0 mM NaCl, 20 mM NaCl and 40 mM NaCl.

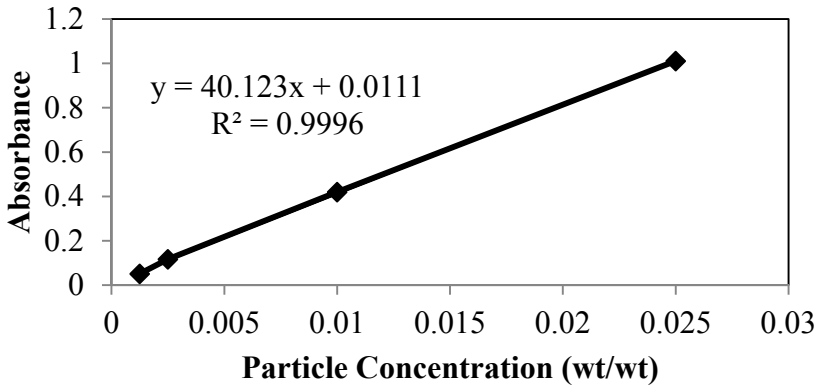

Figure A. 8 Standard curve of 0.25% heated complexes at pH 4.75 measured at 280 nm via UV-Vis. All samples were diluted with pH 4.75 buffer to obtain final concentration